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POSTNATAL ONTOGENY OF THE MOLECULAR EXPRESSION OF INSULIN-LIKE GROWTH FACTOR I (IGF-I) AND TYPE I IGF RECEPTOR IN BOVINE SKELETAL MUSCLE

BY

DARRELL R. PETERSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

ANIMAL BIOTECHNOLOGY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

SPRING 1991

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THE UNDERSIGNED CERTIFY THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED Postnatal ontogeny of the molecular expression of insulin-like growth factor I (IGF-I) and type I IGF receptor in bovine skeletal muscle.

SUBMITTED BY Darrell R. Peterson

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Date: MARCH 20 1991

ABSTRACT

The elucidation of molecular determinants regulating bovine tissue growth may facilitate improvement in either the efficiency or the magnitude of lean production in cattle. Northern blot hybridization analysis was employed to evaluate postnatal ontogeny of insulin-like growth factor I (IGF-I) and type I IGF receptor mRNAs in skeletal muscle within and between 3 strains of cattle displaying differential muscling phenotypes. To accurately interpret specific mRNA signals, a combination of distinct radiolabelled probes and hybridization methodologies were utilized. Through this approach, pilot studies validated the following IGF-I mRNA transcripts: 9.4kb and 7.7kb in bovine muscle and pituitary; 10.3kb, 7.7kb, 3.5kb and 1.1-0.8kb in bovine liver; 7.7kb and 1.1-0.8kb in rat liver. Production of this mitogen by multiple tissues may reflect a localized control of body growth in which autocrine/paracrine agents augment an endocrine homeorhesis. Type I IGF receptor transcripts of 11.0kb, 6.1kb, 4.4kb and 3.1kb were identified within all tissues; however, signal intensities varied in a tissue specific manner. Heterogeneity in transcript species and/or intensity may engender diversity in the capacity of the transcripts to contribute to tissue and/or species specific requirements.

Species of IGF-I and type I IGF receptor transcripts in skeletal muscle showed neither strain- nor age- specific variation over the first 6 months of postnatal development. However, the abundance of all messages, as measured by autoradiographic intensities, gradually increased. The mRNA for these two polypeptides may, thus, be

developmentally regulated at the level of transcription or stability; the identified increase in transcript abundance may represent a mechanism to effect cell responsiveness.

Previous work has provided strong evidence that within a given species, circulating IGF-I levels may well influence the size of individual animals provided comparable environment and suitable nutrition. However, serum IGF-I radioimmunoassay in the two experimental cattle strains manifesting the most disparate body growth parameters, suggests that circulating IGF-I levels need not be positively correlated with body weight within and/or between these two groups. Serum IGF-I levels, therefore, may not necessarily be a physiologic indicator of growth potential in cattle during the first 6 months of postnatal development.

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I.1. INTRODUCTION

When tillage begins, other arts follow.
The farmers, therefore, are the founders of human civilization.

Daniel Webster (Bartlett, 1968)

Animal products are the primary source of dietary protein in developed countries. In Canada, livestock represents one of our largest agricultural sectors accounting for 27% of $19.5 billion in annual gross farm cash receipts. The gross receipts for beef cattle alone amount to ~$3.9 billion annually (Statistics Canada, 1990). Beef cattle are essentially produced for the lean or muscle content of their carcass. Producers are concerned with manipulating growth and composition with intention to increase the proportion of muscle, decrease the proportion of bone and optimize the proportion of fat. Strategies to favorably alter the body composition of livestock promise to increase farm output while decreasing capital costs through higher productivity.

The genetic manipulation of microbes, plants and animals has served human design for thousands of years. The term biotechnology has been used to define this manipulation, broadly encompassing both conventional breeding practices as well as genetically augmented transformations. Stemming from relatively long reproductive intervals, the enhancement of agriculturally desirable phenotypes by conventional intervention is a somewhat leaden exercise. Changes in production parameters could possibly be accomplished more expeditiously through engineered transformations. Presently, growth can be enhanced by introducing genes coding for polypeptides mediating its regulation, fused to ubiquitous promoters, into

the germ line of animals (Quaife et al., 1989). With this technology, the beef sector can forsee the development of transgenic livestock that are leaner, more heavily muscled and reach market size and weight more quickly than traditional breeds.

As with all species, cattle manifest a variable sex, breed, and individual influence in muscling and rate of growth (Berg, 1984). It is unlikely that any one gene is responsible for these phenotypic differences; rather, many genes and their alleles code for initiating and intermediary molecules which act in biochemical pathways directing tissue development. An understanding of the mechanisms of growth enhancing factors, and their utilization in a time and space dependant manner, could permit improved accuracy in the selection of breeding animals and/or the genetic transformation of embryos with important alleles. As such, this investigation was undertaken to further elucidate molecular determinants promoting tissue growth.

The purpose of the ensuing literature review is three-fold, clarifying and detailing: a.) cattle strains comprising the investigative study group, b.) postnatal growth characteristics of bovine skeletal muscle concomitant with a summary review of skeletal muscle development, c.) an understanding, in brief, of the molecular biology of the somatotropic axis and the influence of this axis on growth.

I.2. CATTLE POPULATIONS

Clearly, there are many genetic and non-genetic differences between

cattle breeds which may affect growth. Investigations into factors regulating body growth must attempt to minimize these differences, and would ideally be implemented in populations derived from a single gene pool. Owing to the inaccessibility of such a population exhibiting significant variation in muscling phenotype, this investigation chose to examine genetic subgroups of disparate body size within closely related breed strains. The animals used in the following series of studies were obtained from either the double-muscled (DM), beef synthetic (SY) or pee-wee (PW) herds maintained at the University of Alberta beef research ranch, Kinsella, Alberta. In 1960, the Department of Animal Science initiated a study into hybrid cattle performance indices with the objective to investigate the importance of hybridization for improving animal strains. The initial hybrid population has diverged since its inception and been expanded so that there are now the 3 unique strains of animals listed above. Once established, each of the foundation populations was closed to the introduction of outside genetic material. As the ranch is managed as a low cost commercial operation, and all management and environmental factors have remained constant, it is assumed that the extremes in growth phenotype displayed between strains is the culmination of many generations of indirect selective breeding for growth regulating genes.

The beef synthetic herd was originated primarily as a composite of Charolais, Angus and Galloway. Charolais was chosen for its growth and leanness traits, Galloway for its hardiness and Angus for its maternal and adaptability traits. The males were selected on the basis of high growth

rate to one year of age, and the females for regular reproductive characteristics. As an approximate percent of current composition, Angus contribute 37%, Charolais 34%, Galloway 21% with 8% coming from assorted other breeds. The contemporary SY line produce progeny that, on average, are 47 kg heavier at birth and 200 kg heavier at 1 year (males), than were their progenitors (Berg et al., 1986). Beef synthetics typically display an average daily gain (ADG) of ~2 kg/day.

The pee-wee population resulted from the crossing of a variety of animals, derived from the initial hybrid population, selected for minimum growth rate in the first year. Present yearling body weights of the PW cattle are approximately 1/2 those of the beef synthetic. The genetic differences between PW and SY animals may be related, but not limited to: founder effect, selection, and/or mutation. The PWs viability as a productive meat species is hampered by their low mature body weight, their slow rate of growth and their inherent nervous constitution.

The DM population was started in 1967 with three extreme muscled DM bulls; one Angus, one Charolais and a Limousin-Hereford, plus an extreme-muscled bull from the Kinsella synthetic population. Initially, cows bred to these DM bulls were normal muscled crossbreeds from the SY line. As of 1976, the herd has been maintained as a closed population open only to extreme muscled animals arising in other Kinsella herds. The breed composition at present is approximately 47% Angus, 14% each of Charolais, Galloway and Hereford, and 11% from other breeds.

Double muscling is a genetic condition characterized by a generalized skeletal muscle hypertrophy. It is manifest by a decreased fat deposition (sub-cutaneous, inter and intramuscular), a lower percentage of muscle in the thorax and distal limbs, and a higher percentage of muscle in the hip and stifle region as compared to industry Herefords (HE) (Berg and Butterfield, 1978; Berg and Shahin, 1983). Heavily muscled DMs have been shown to have increased hypertrophy in regions of high priced cuts, particularly in the large muscles of the proximal pelvic limb (Shahin and Berg, 1983). At the same total side muscle weight, DMs have a higher muscle to bone ratio than do synthetics (6.5:1 DM, 5.6:1 SY) (Berg and Shahin, 1983). Yield of muscle as a percent of body weight also ranks DM higher in comparison to SY with a muscle relative to liveweight ratio of 41% for DM and 39% for SY (Berg and Shahin, 1983). The genetic differences between DM, and SY and PW strains, are related, but not limited to: founder effect, selection, introduction of outside genetic material, and/or mutation.

The mode of inheritance of the DM trait has been described as being "a single incompletely recessive gene with variable expression mediated by modifying gene products" (Basarab, 1981). The underlying biochemical abberations responsible for the DM syndrome are unknown. Some investigators have suggested that the primary defect lay in an endocrine imbalance in turn altering cellular differentiation, growth, and metabolism (Strath et al., 1982). Others have tabled evidence to suggest that an abnormality may be expressed at the cell membrane (Basarab et al., 1980). Alterations of membrane structural integrity result in a proposed

disruption of neuromuscular contact propagating hyperplasia/hypertrophy.

Double muscled animals may provide a viable opportunity to increase the yield of edible lean from beef; however, DMs are beset with associated fitness problems. Stress susceptibility, early mortality, poor reproductive performance and a relatively short productive lifespan contribute to the negative realization of DM productivity (Strath et al., 1980). A genetic understanding of the DM condition may forsee a means to capitalize on its productive traits while avoiding any non-productive repercussions.

SY and PW strains originated from the same initial hybrid population less than 25 years ago. DM animals represent a closely related breed group manifesting an inherited muscular hypertrophy. Each population presently express differing rates of muscle growth postnatally. On these statements, this investigation advances that the 3 herds are potentially useful for studies of bovine muscle growth regulation, and moreover, that comparisons between them may allow further clarification of molecular determinants contributing to variable growth rates.

I.3. SKELETAL MUSCLE DEVELOPMENT

The ensuing section outlines the postnatal development of bovine skeletal muscle, briefly details skeletal muscle cell development, and describes factors which are known to affect muscle growth. In particular, the influence of hormones and growth factors on muscle growth, with special reference to the insulin-like growth factors, is raised.

During the early postnatal period, domestic cattle exhibit the greatest change in their relative weight occurring before the sixth month of age. A considerable change is noted even within the first three months (Butterfield, 1966; Butterfield and Berg, 1966a,b). As the animal approaches maturity, the rate of weight change begins to slow. During the prepubertal period, the bovine carcass shows differential growth involving its major tissues - bone, muscle and fat (Berg, 1984; Berg and Butterfield, 1979). Each can be respectively classified on a whole animal basis as: early developing, intermediate developing and late developing (Berg and Butterfield, 1979). The relative proportion of bone decreases progressively after parturition until the stable adult composition is attained. The intermediate developing muscle starts its proportional increase at birth stablizing at maturity. Subsequent to this stabilization, the whole animal proportion of adipose tissue progressively increases as the animal continues to age.

The concept of functional demand influencing muscle growth during development has been proposed (Berg and Butterfield, 1978). This describes the adjustment of muscle weight distribution in the growing calf as an acquired genetic pattern. Butterfield and Berg, (1966a,b) have defined an "*impetus*" for the description of muscle growth. Those muscle groups growing at a faster relative rate than total muscle, are considered *high impetus*. *Average impetus* muscles grow at the same relative rate as total muscle, and *low impetus* clusters grow at a lower relative rate than total. Some muscles are found to have a diphasic growth pattern changing during postnatal development. Much of the economically important muscle

mass is of this class, displaying a retrograde change in growth impetus before muscle weight at birth has doubled (Butterfield and Berg, 1966a,b).

Proliferation of muscle cells occurs predominantly before birth. During ontogenetic development, embryonic precursors engender a committed stem cell, the myoblast (White and Esser, 1989). Eventually cell populations cease dividing and start to align, subsequently fusing to give rise to myotubes. The appearance, accumulation and sarcomeric assembly of muscle specific isoforms of the contractile proteins characterize terminal differentiation. As the myotube matures, its nuclei migrate peripherally and fascicular innervation occurs. A second mononuclear cell, the satellite cell, is derived from a population of myoblasts which do not fuse or align. It is carried peripherally along with the myotube nuclei during differentiation. Satellite cells are characterized by their location outside of the muscle fiber plasma membrane but within the basal lamina (White and Esser, 1989). Their heterochromatic nucleus is easily distinguished from the euchromatic myonuclei. Satellite cells function as reserve committed muscle stem cells providing functional nuclei to growing muscle cells (White and Esser, 1989). In adult skeletal muscle, they are normally mitotically quiescent but can be induced into mitotic activity by appropriate stimuli.

Postnatal enlargement of muscle fibers is accomplished essentially by increases in length and diameter. Interestingly, a comparison of normal Hereford and DM animals shows that cross-sectional diameters of the muscle fibers in the two genotypes do not differ. The primary factor resulting

in the DM hypertrophied state is prenatal hyperplasia. DM animals develop more muscle fibers as early as the first third of gestation. Many of these extra fibers are of the α type, which in adult correspond to anaerobic fast twitch species (Ashmore et al., 1974).

Control of somatic muscle growth is influenced by genetic predisposition, plane of nutrition and disease state (Berg, 1984). Coordinating with these integrated systems, a cascade of circulating hormones emanating from the hypothalamus, pituitary, liver and other tissues, modulates tissue development. Growth results when the anabolic versus catabolic equilibrium favors net anabolism. Thyroid hormone, insulin and growth hormone (GH) are the principal anabolic promoters stimulating muscle growth at the whole body level (Florini, 1987). Thyroid hormone encourages cellular metabolism by translational and transcriptional induction of enzymes necessary for the synthesis of proteins, and the oxidative breakdown of fats and carbohydrates. Insulin stimulates the deposition of glycogen, triglycerides and protein consistent with its function in promoting nutrient storage (Fulks et al., 1975). Growth hormone directs a general cellular hypertrophy manifest by an increase in a variety of transcriptional pathways (See I.4.1). At the cellular level, the primary anabolic hormones include the insulin-like growth factors, epidermal growth factors and fibroblast growth factors (Bischoff, 1986; Schoelne and Zapf, 1982). The presence or absence of these mitogens during G_1 determines whether myoblasts enter S-phase and continue through another cell cycle or stop in G_1 and commit to differentiation (Ewton and Florini, 1980; Turner et al., 1988).

Constituting a catabolic force in the growth equilibrium, glucocorticoids mediate an antagonistic role to anabolism. The presence of glucocorticoids: increases the production of intracellular proteolytic enzymes (Florini, 1987), decreases DNA and protein synthesis (Florini, 1987), and reduces amino acid uptake by the muscle (McGrath et al., 1987).

Manipulation of factors promoting muscle growth to optimize production indices in domestic cattle, has thus far focused largely on the role of circulating growth hormone. However, it has become increasingly clear that GH may have a limited role as an anabolic promoter. It was initially thought that all the actions of this peptide were exerted directly on target tissues. Nevertheless, in 1957, Salmon and Daughaday discovered that the effect of GH to stimulate incorporation of sulphate into rat cartilage was not a direct one, but rather was mediated through some uncharacterized endocrine determinant. The term somatomedin, a generic reference for serum derived peptides mediating GH action, was originally coined to describe these agents. Currently, and in part due to their structural and functional similarity to insulin (Rinderknecht and Humbel, 1978a), the somatomedins have become known as the insulin-like growth factors (IGFs). Two classes of IGF have been isolated on the basis of physiochemical data. They are known respectively as insulin-like growth factor I (IGF-I), and insulin-like growth factor II (IGF-II) (Hill et al., 1984). Growth hormone appears to be a dominant endocrine influence on the expression of IGF-I (Johnson et al., 1989; Murphy and Friesen, 1988; Schalch et al., 1979), with nuclear run-on assays clearly showing regulation occurring at the transcriptional level (Mathews et al., 1986).

In hepatic tissue, IGF-I transcription is biphasic in nature with a dramatic increase shortly after GH ligand-receptor interaction, followed by a decline, and subsequent reaccumulation of IGF-I message within 24 hours (Johnson et al., 1989). This twofold response appears to be due to the acute, transitory down regulation of hepatic GH receptors following GH stimulation. The somatomedin hypothesis of Salmon and Daughaday (1957), as described, has been extensively confirmed and recently extended to include, along with the originally recognized endocrine role, autocrine and paracrine capacities (Clemmons and Van Wyk, 1985; D'Ercole et al., 1984).

It is presently acknowledged that GH exerts a variety of anabolic effects, some mediated directly (Quaife et al., 1989), whereas its primary actions stem from self-dependent synthesis and release of IGF-I. In skeletal muscle, IGFs stimulate cellular proliferation and differentiation, as well as exert insulin like effects on intermediary metabolism (Ewton and Florini, 1980; Tollefsen et al., 1989a). Ewton and Florini, (1980) have proposed that the early growth of muscle is stimulated primarily by these polypeptides, and that as hypertrophy replaces hyperplasia as the priority in growth, the tissue becomes responsive to growth hormone as well. The IGFs would appear to embody a more direct influence on growth and development than GH. Therefore, this thesis proposes an examination of IGF influence on muscle growth with the intent to forward these agents as molecular determinants facilitating the phenotypic variation exhibited between PW, DM and SY cattle strains.

I.4. MOLECULAR BIOLOGY OF THE SOMATOTROPIC AXIS

This section describes specifics regarding the molecular biology and physiology of the primary members of the somatotropic axis. Special attention is focused on the characteristics of insulin-like growth factor I and its cell surface receptors. Most of the information cited has been obtained through studies in man and rodents (Clemmons, 1989; Daughaday and Rotwein, 1989; Froesch et al., 1985; Froesch and Zapf, 1985; Han et al., 1988). From data specific to domestic cattle (Fotsis et al., 1990; Honnegger and Humbel, 1986), it is presumed that there would be little significant difference between bovine, human and rodent somatotropic characteristics. However, cautious inference is applied when bovine details are not known.

I.4.1. GROWTH HORMONE

Multiple cis- and trans-active elements exhibit synergistic interaction in determining transcriptional activation of the growth hormone gene. This activation occurs in constituent acidophilic somatotrophs of the anterior pituitary (adenohypophysis). The 5′ flanking region of the GH gene contains a trans-sensitive TATA box with binding sites for pituitary specific transcription factors. In addition, the promoter also contains cis-elements modulating transcription in response to hormonal signals (Karin et al., 1990). Secretion of GH is regulated by a dual system of hypothalamic-hypophysiotrophic hormones; growth hormone releasing factor (GRF) stimulates GH release, while somatostatin inhibits GH expression. Release of these peptides from the hypothalamus is in turn modulated by a network of monoaminergic neurons dependent on

excitation under the influence of information present in the limbic system, as well as the brain stem (Hammerman, 1989; Martin, 1983).

Secretion of the 191 residue single chain GH polypeptide occurs by exocytosis with fusion of the secretory granule membrane to the plasma membrane. In ruminants, this secretion is pulsatile in nature being both asynchronous and episodic (Gluckman and Brier, 1987). The physiological significance of such pulsatility remains to be elucidated; however, sexually differentiated patterns of GH expression are documented (Isgaard et al., 1988; Martin, 1983). Masculine plasma GH patterns are evidenced by higher pulse amplitudes and lower baseline values, while female patterns display a more constant expression omitting the extremes in fluctuation. Induction of a masculine pattern in female rats is associated with enhanced body growth. Establishing a female profile in males leads to a much reduced body mass gain (Isgaard et al., 1988). Data such as this lends credence to the importance of GH pulsatility influencing biological mass.

While growth hormone's effector actions in vivo are complemented by concurrently present peptidergic and steroidal inputs (Murphy and Friesen, 1988), GH is known to regulate a number of anabolic processes associated with muscle cell growth (for review Florini, 1987) including: protein and RNA synthesis, and the activity of RNA polymerase I and II. In cell culture, growth hormone's actions to regulate glucose metabolism are exerted directly on sensitive cells, whereas stimulation of somatic growth is indirect, mediated through the IGFs (Hammerman, 1989). Both GH and

IGFs exert negative feedback regulation on pituitary GH expression by increasing somatostatin release and decreasing GRF secretion (Fagin et al., 1988).

I.4.2. SOMATOMEDINS/INSULIN-LIKE GROWTH FACTORS

Insulin-like growth factor I and insulin-like growth factor II have a 70% amino acid sequence identity with each other and almost a 50% identity with insulin (Rinderknecht and Humbel, 1978a; Shimatsu and Rotwein, 1987; Zapf et al., 1984). They both contain an A domain that is homologous to the A chain of insulin and a B domain homologous to the B chain of insulin. Insulin, IGF-I and IGF-II each contain a C region which is retained in the IGFs but lost in the processing of mature insulin. In addition, IGF-I and II contain a D domain extending from the carboxyl terminus of the C chain, and an E domain that is cleaved during processing of the mature peptide. One notable difference between IGFs and insulin is the presence of an associated specific carrier protein complexed to circulating IGFs (Binoux et al., 1986). Little, if any free hormone, is present in serum or plasma.

As the basis for the biological properties of IGFs rests in their structural homology with insulin, it is not surprising that each elicit overlapping effects. The ancestral relationship between insulin and IGFs (Bell et al., 1985) suggests a complementary anabolic role wherein IGF appears to be a major anabolic hormone for growth and differentiation, while insulin acts in a complementary fashion acutely providing and regulating the energy for these processes (Zapf and Froesch, 1986). The

ubiquitous cellular requirements for the IGF polypeptides suggest that their biological actions also fulfill specific needs that are not met by insulin or other hormones/growth factors. They exert pleiotropic effects on diverse cell types through interactions with specific high affinity cell surface receptors (Clemmons, 1989). They regulate a variety of metabolic and transport processes in some cells and act as mitogens for many others (Ballard et al., 1989; Hill, 1988). The dominant actions of IGFs appear to depend in large part on the concomitant influence of other competence and inhibitory factors (Van Zoelen, 1987). Within this complex network, IGFs are believed to act as progression factors controlling the passage of competent cells through the G_1/S boundary (Clemmons, 1989; Yang and Pardee, 1986). The ensuing actions of IGFs in vitro can be distinguished on a temporal basis. Initially, acute metabolic effects occur within seconds of induction (Tollefsen et al., 1989a). These effects include a stimulation of glucose and amino acid transport, a rapid increase in the rate of polypeptide chain initiation, a general cellular anabolism, and an inhibition of catabolic processes (Ballard et al., 1989; Poggi et al., 1979). Longer term anabolic effects include IGFs regulation of transcriptional events necessary for DNA and RNA synthesis (Monier and Le Marchand-Brustel, 1984; Yang and Pardee, 1986; Zorzano et al., 1988). Overall, IGFs exert mitogenic and/or differentiative actions on cells of all embryonic origins.

The source of circulating IGF-I and IGF-II was traditionally believed to be the liver. The strong correlation between hepatic IGF content and circulating IGF levels supports this hypothesis (Lund et al., 1986).

However, recent investigations have accumulated evidence indicating that the genes and/or peptides of IGFs are expressed in multiple organs and tissues (Brown et al., 1986; Han et al., 1987a,b; Lowe et al., 1988; Murphy et al., 1987b; Zangger et al., 1986). Furthermore, specific receptors for IGF-I and II have been identified in several mammalian cell types (Beguinot et al., 1985; Burant el al., 1987). It is proposed that extrahepatic expression of IGF-I may account for 45% of the total circulating IGF-I levels with hepatic expression contributing the remaining 55% (D'Ercole et al., 1984). Liver appears to have a steady state messenger RNA level 15 times that of any nonhepatic source (Lowe et al., 1988), being sufficient to provide for turnover of plasma IGF-I (Roberts et al., 1986).

The presence of insulin-like growth factors within a tissue can originate from exogenous sources, as from the liver, or endogenously, as from itself. Locally produced IGFs can act on the cell types they emanate from (autocrine) or on adjacent cells in the tissue (paracrine). Tissue level IGF production could adapt to subserve special demands posed by a local microenvironment. These demands may include normal growth, neoplastic growth (Hoppener et al., 1988; Williams et al., 1989) or regenerative processes such as wound healing and tissue remodelling (Hanson et al., 1988; Jennische and Hanson, 1987). Autocrine/paracrine short range stimulatory loops appear to be present from conception onwards. Preimplantation embryos grow and differentiate in the absence of exogenous factors; accordingly, endogenous mitogens such as IGF may contribute to the development of the embryo during the first 7 or 8

cleavage divisions (Rappolee et al., 1988). Postnatally, autocrine/paracrine IGF could play a role in tissues where anatomical barriers, such as the blood brain barrier, limit entry of circulatory growth factors (Daughaday and Rotwein, 1989). Locally produced IGFs may contribute to metabolic control in a manner supportive of the stimulatory effects of an endocrine and/or nervous message.

The mechanisms of autocrine growth stimulation are still unclear, particularly with respect to how and where receptor-ligand interactions take place. The growth factor and its receptor may be synthesized and transported separately to the plasma membrane, subsequently recognizing each other extracellularly. Alternatively, this interaction may occur cytoplasmically, guided by some form of cytoskeletal control (Van Zoelen, 1987). Regardless of the pathway utilized, it is suggested that IGFs of autocrine, paracrine and endocrine origin influence tissue growth and development.

I.4.3. INSULIN-LIKE GROWTH FACTOR I

The human IGF-I (hIGF-I) gene is present once per haploid genome (Tricoli et al., 1984). Its discontinuous coding region of over 85kb contains 5 exons interrupted by 4 introns (Rotwein et al., 1986). Flanking the gene are encoded amino and carboxyl terminal peptides and extensive 5′ and 3′ untranslated regions (UTRs). The genomic location of IGF-I and its functional homology to insulin, lends itself to the hypothesis that IGF-I and insulin diverged from a common ancestral gene (Bell et al., 1985). According to this premise, there was a gene

duplication roughly 600 million years ago, that lead to the evolution of proinsulin and a primitive IGF molecule. A second duplication and simultaneous translocation placed the primordial IGF-I sequence on a separate chromosome whereas IGF-II remained closely linked with the gene from which it arose.

The IGF-I gene encodes a heterogenous group of messenger ribonucleic acids (mRNA) arising from alternative splicing of sequences corresponding to 5′ UTR, 3′ UTR and E peptide domains (Fagin et al., 1989). For bovine and human IGF-I, two potential translation products exist, IGF-IA and IGF-IB (Rotwein, 1986; Honneger and Humbel, 1986). The two precursors are synthesized from distinct mRNAs which are produced by alternative splicing of the primary IGF-I gene transcript (Fotsis et al., 1990; Rotwein et al., 1986) IGF-IA mRNA contains exons 1,2,3 and 5, while IB encodes 1,2,3 and 4. The mature plasma IGF-I is encoded within exons 2 and 3. IGF-IA and IB are identical from their aminoterminus to a lysine residue 16 codons beyond the IGF-I coding sequence. This 134 residue peptide includes the co-translationally cleaved signal peptide (amino acids 1-48, exons 1 and 2), the 70 residue IGF-I molecule (amino acids 49-118, exons 2 and 3) and the amino acids comprising the initial part of the E domain (exon 3). Exons 4 and 5 transcribe subsequently divergent E extension peptides, termination codons, 3′ UTRs, and polyadenylation signals. IGF-IA contains an additional 19 amino acids encoded on exon 5 giving a total length of 153 residues. Exon 5 is located ~1.5 Kb 3′ to exon 4, is 344 nucleotides long, and contains multiple polyadenylation sites within a 284 base 3′ UTR. Three of these poly A signals are of consensus type (5′-AAUAAA-3′)

showing addition signals 13-21 nucleotides 5′ to the site. The open reading frame of IGF-IB is 195 residues long including the additional 61 amino acids encoded on exon 4. Exon 4 is located ~1.5 Kb 3′ to exon 3, is 515 nucleotides long and is followed by 329 bases of 3′ UTR containing only one near consensus polyadenylation signal.

In contrast to human and bovine IGF-I, rodent IGF-I cDNAs reveal species specific distinctions. Although rat IGF-I also contains IA and IB transcripts (Murphy et al., 1987a), IGF-IB mRNA is generated by the addition of a 52 nucleotide "mini-exon" during processing. This insert, spliced between exons 3 and 5, leads to a change in translational reading frame and stop codon of regions beyond the mature coding sequence. Rat IGF-IA or IB may contain any of three alternative 5′ UTRs termed class A,B and C. Each of these regions contain multiple upstream in-frame initiation codons, any of which generate heterogeneity in pre-pro IGF-I N-terminal peptides (Roberts et al., 1987a). It is suggested that the secondary structure of this area may affect mRNA stability, translatability and/or intracellular targeting of the polynucleotide (Daughaday and Rotwein, 1989; Lowe et al., 1987). The choice of 5′ UT regions and the presence or absence of the 52 base "mini-exon" are independent events (Lowe et al., 1988). As such, at least six species of IGF-I mRNA are able to be transcribed. Besides the 5′ and carboxyl terminal E domain variation described, further heterogeneity may be introduced by use of alternate polyadenylation sites, 5 of which have been characterized (Daughaday and Rotwein, 1989).

In all species investigated, Northern hybridization analysis detect more IGF-I transcripts than can be accounted for by the processing steps described. The smallest of the cited transcripts, ~1.0-0.7kb, is of sufficient length to encode for pre-pro IGF-I (Roberts et al., 1986). As the largest mRNA class of 9.0-7.0kb is not processed into smaller species (Lowe et al., 1989), the significance of the larger species is unknown. Sequence and translational capacity of these large molecular weight mRNAs also remains undetermined. Differential regulation of large transcripts is suggested since not all transcripts appear to be affected equally by modulators of IGF expression. (Lowe et al., 1989; Roberts et al., 1987b). This regulation appears to be both tissue specific and age specific. Biochemical analysis has revealed that changes in the levels of different 5′ UTR and E peptide variants in the rat are not due to differential stabilities but to changes in transcription rate (Lowe et al., 1989).

A comparative review of the literature (Figure I.1, I.2) shows that some reported IGF-I transcript size variability may result from discrepancies in molecular weight estimation. Moreover, the authenticity of some IGF-I signals may be suspect due to the stringency of hybridization conditions employed. Presumed non-specific hybridization presents a problem to investigations interpreting Northern blot analysis of IGF-I mRNA expression. In order for this present study to qualify authentic IGF-I transcripts, a methodological validation of the specificity of detected signals will be required (See Chapter II).

The various structural domains of insulin-like growth factor I are

highly conserved across bovine (Fotsis et al., 1990; Honneger and Humbel, 1986), human (Rotwein, 1986), rat (Roberts et al., 1987b) and mouse (Bell et al., 1986). This conserved homology indicates that most sequence differences between species probably alter little the physiological properties of the growth factor. The RNA sequence of mature bovine IGF-I (bIGF-I) is found to be identical to that of mature human IGF-I (hIGF-I) (Fotsis et al., 1990). The human pre-pro molecule encodes a 130 amino acid polypeptide of molecular weight 7,646 (Rotwein et al., 1986). It undergoes proteolytic processing at both ends to generate a mature 70 residue growth factor. Mature IGF-I is a basic protein containing three disulphide bonds endowed with an isoelectric point (pI) around 8.3 (Clemmons, 1989; Rinderknecht and Humbel, 1978a).

The amino acid sequence of the mature IGF-I peptide of rat shows a number of differences from the human/bovine peptide. Although these substitutions range from neutral to conspicuous, none occur at positions which would necessarily alter the predicted overall tertiary structure of characterized active domains (e.g. receptor or carrier protein binding sites) (Bayne et al., 1989; Clemmons, 1989; Konishi et al., 1989; Roberts et al., 1987b).

Unlike most hormones, which are stored in endocrine glands inherent with sophisticated controls over their secretion, IGF-I is secreted as it is produced. Hence, expression appears focused on transcriptional capacity. Although growth hormone is the best characterized regulator of IGF-I transcription in multiple tissues (Fagin et al., 1988; Hammerman,

1989; Hynes et al., 1987; Isgaard et al., 1988; Mathews et al., 1986), additional signals also influence its expression. In hepatic tissue of the postnatal rat, where substrate availability remains an important determinant of growth, insulin regulates not only IGF-I but also inversely, transcription of the small IGF carrier protein gene (Boni-Schnetzler et al., 1989). When insulin levels are low, transcription of IGF-I is also low while binding protein expression is high. This combination succeeds in drastically reducing the insulinomimetic actions of free IGF-I. The nutritional status of an animal can also regulate IGF-I expression through a coordinated regulation of growth hormone. In the bovine, during periods of limited nutrient availability, GH levels increase thereby mobilizing energy from adipose tissue to satisfy metabolic needs (Breier et al., 1988; Gluckman and Breier, 1987). Concurrently, the concentration of hepatic growth hormone receptors decrease; this is linked to a reduction in plasma IGF-I concentration and a fall in its mitogenic influence. Expression can moreover be regulated by non-hormonal/neural determinants such as cell density and cell cycle timing (Clemmons and Van Wyk, 1985).

Although IGF-I is not the only determinant involved in the regulation of growth, a positive correlation appears to exist with respect to IGF-I secretory capacity and growth. Serum IGF-I levels are age and sex dependent and show a positive correlation with birth weight in man (Furlanetto and Cara, 1986). Circulating levels of IGF-I are correlated with body size/growth velocity in domestic cattle (Lund-larsen et al., 1977; Rumsey and Elasser, 1987), chickens (Hoshino and Wakita, 1982), and

swine (Buonomo et al., 1987). A similar relationship has been noted in dogs (Eigenmann et al., 1984a,b) and man (Gnurmelen et al., 1986; Merimee et al., 1982; Rieu, 1986). Furthermore, substantial responses in growth rate and weights of rodents occurs after selection for, or against, plasma levels of IGF-I (Blair et al., 1988; McGrath et al., 1987). The production of this polypeptide does not simply represent an epiphenomenon that reflects growth; rather, expression of IGF-I appears causally linked to stimulation of cellular replication. Postnatal growth in rats has been demonstrated to be contingent on IGF-I activity (Hall et al., 1988).

There is some evidence suggesting a degree of genetic determination for plasma IGF-I concentrations (Eigenmann et al., 1984a; Furlanetto and Cara, 1986). Levels of IGF-I in three distinct breeds of poodles differing in adult size (toy, miniature and standard) show that body weight is positively correlated with IGF-I levels rather than with GH. As well, a good interaction has been described between weight gain and IGF-I expression in select tissues (Murphy et al., 1987b). This in turn provides further support for autocrine/paracrine contributions mediating growth.

As described earlier, IGF-I appears to have evolved from the insulin gene. It is possible that the proposed translocation of the IGF-I gene inserted it near enhancer/promoter elements driving postnatal growth. IGF-II remained under the regulation of its own specific cis- and trans-activators. This resulted in a developmental separation of IGF-I and II in terms of their mitogenic capacity. Although the function of IGF-II is

not as well characterized, it has been implicated as a cellular progression factor preferential for prenatal development that is replaced by IGF-I at parturition (Brown et al., 1986; Daughaday et al., 1982; Froesch and Zapf, 1985). This developmental regulation of expression remains elusive in its generation and purpose.

I.4.4 INSULIN-LIKE GROWTH FACTOR II

Pre-pro IGF-II, also known as multiplication stimulating activity (MSA) (Zapf and Froesch, 1987), is a 180 amino acid protein of molecular weight 20,100. Subsequent proteolytic modification generates the mature 67 amino acid (7,471 daltons) single chain polypeptide (Rinderknecht and Humbel, 1978b), that displays functional conservation across species. Bovine IGF-II differs from human IGF-II by only 3 amino acids and differs from rat IGF-II by 5 residues (Bell and Merryweather, 1984; Zapf and Froesch, 1987).

Whereas IGF-I gene expression is primarily regulated by GH, IGF-II expression is fundamentally governed by placental lactogen (Caufriez and Copinschi, 1986; Furlanetto and Cara, 1986), aligning it well as the primary mediator of prenatal growth. Evidence exists for developmental specific chromatin structure and promoter/enhancer regions, 5′ to the IGF-II gene, that generate alternative primary transcripts (Hoppener et al., 1988). Post-transcriptional processing is proposed as the underlying mechanism generating further transcript heterogeneity. Rodent liver, muscle, and lung tissue hybridize with six different mRNAs of size 6.0kb, 4.0kb, 2.8kb, 2.2kb, 1.7kb and 1.2kb (Brown et al., 1986). Functional

roles for these variants are yet to be elucidated.

I.4.5. INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

In all interstitial and extracellular fluids that have been analyzed, less than 1% of the insulin-like growth factor exists as free polypeptide (Blum et al., 1989). The remaining 99% circulates non-covalently complexed to specific high affinity carrier proteins (binding proteins (BP)) (Clemmons et al., 1989; Froesch et al., 1985; Zapf and Froesch, 1987). Two predominant IGF/BP conjugates have been identified. The largest, and that constituting the major proportion (~80%) of circulating IGF, is a 150kda trimer consisting of a polypeptide binding protein (a β subunit also known as BP-53), an acid labile component (an α subunit) and the growth factor ligand itself (Hintz, 1984). This substantial complex appears to be largely restricted within the vasculature suggesting a reservoir function (Hall et al., 1988; Hill et al., 1989). A minor proportion of IGF (~19%) circulates as part of a complex with a molecular weight being variously estimated between 45kda and 16kda (for review, Froesch et al., 1985). This form, consisting of a simple noncovalently bound polypeptide ligand and a BP subunit (33% homology with BP-53), is ubiquitously distributed in all extracelluar fluids circulating at levels 100 fold lower than the 150kda BP. (Czech et al., 1989). The physically heterogenous family of low molecular weight BPs each contain a core protein of 198 amino acids, with the differences in molecular weight being attributed to various carbohydrate moiety extensions (Clemmons et al., 1989). Supporting the hypothesis that IGF-I substitutes for IGF-II as a primary growth stimulus postpartum, experimental evidence in rodents has

revealed a progressive change with age in affinity of the BPs for the two IGFs (Binoux et al., 1986; Canover and Lee, 1990). In the fetal circulation there is a higher proportion of IGF-II and the lower molecular weight BPs, while postnatally, there is a higher proportion of IGF-I and the 150kda BP.

The exact physiological role of circulating IGF-BPs remains unclear. Present evidence suggests that they are not mere carriers but that they play an active and complex role in IGF physiology. BPs function to inactivate the acute insulinomemetic effect characteristic of free IGFs (Binoux et al., 1986; Buonomo et al., 1987). Mitogenic vigour appears to be induced only by non-complexed polypeptide. A recently isolated aminoterminus truncated derivative of IGF-I, (des-(1-3)-IGF-I), has normal IGF-I activity but at a 50 fold increased potency (Ross et al., 1989). This increase is not associated with a higher affinity for IGF receptor, but instead, is correlated with poor binding to carrier proteins.

Binding proteins may dictate specificity and rates of delivery of IGF to tissues (Hintz, 1984). The smaller molecular weight BPs have been proposed to act as a shuttle for IGFs from the storage, 150kda form in the circulation, to target cells (Hall et al., 1988). Furthermore, some members of this BP family appear to be endowed with an RGD (Arg-Gly-Asp) adhesion sequence near their carboxyl terminus (Hill et al., 1989). This tripeptide has been detected in both human and bovine studies and is postulated to allow interactions of the BPs with extracellular matrix protein receptors and/or integrins (Hill et al., 1989). It is conceivable

that this feature may generate a target tissue micro-reservoir of autocrine, paracrine or endocrine IGF, enhancing the growth factors mitogenic capacity (Elgin et al., 1987).

IGF binding proteins prolong metabolic half life (T 1/2) of their associated ligand. The 150kda complex has a longer T 1/2 than the 45kda, and both have significantly longer T 1/2 than does free IGF (Buonomo et al., 1987; Hintz, 1984). The long T 1/2 of the 150kda complex (upwards of 20 hrs) explains the remarkable consistency of plasma IGF levels throughout a 24 hr period (Blum et al., 1989). It is possible that the influence of IGFs requires a steady state level of receptor occupancy to provide optimum mitogenic stimulus.

In contrast to certain other growth factors (eg. epidermal growth factor, transforming growth factor -β), IGFs and their BPs are not products of a proteolytically modified single gene transcript (Miyazano et al., 1988). IGFs and their BPs are encoded by separate genes (Clemmons et al., 1989; Rotwein et al., 1986). Induction of BPs by factors such as GH (Clemmons et al., 1989; Hintz, 1984), IGFs (Clemmons et al., 1989; Hill et al., 1989), and/or other metabolic indicators (Busby et al., 1988), coordinates release of BP and IGF and may contribute to regulating IGF activity. Several cell types have been shown to secrete BPs following stimulation by IGF-I (Hintz, 1984). The cellular distribution of this expression is similar to that for the expression of the IGF ligand. As such, it is feasible that acute regulation of IGF-I activity may partially occur not only through mediation of ligand expression levels, but

additionally through carrier protein governance.

I.4.6. INSULIN-LIKE GROWTH FACTOR RECEPTORS

Insulin-like growth factors exert their effects through interaction with a family of high affinity cell surface receptors that extract the ligand from its carrier protein via, as yet, undetermined mechanisms. Competitive binding studies (Ballard et al., 1988), and experiments (Clemmons, 1989; Ullrich et al., 1986) examining these receptors, have described three individual, yet related species. The IGF type I receptor binds IGF-I > IGF-II > insulin; the IGF type II receptor binds IGF-II > IGF-I, and the insulin receptor binds insulin > IGF-I > IGF-II. Both insulin and type I receptor bind their respective ligands with high affinity (k_d=1nm) (Czech et al., 1989; Verland and Gammeltoft, 1989). The cross-reactivity shown by IGF-I and insulin ligands for each others receptors results from the partially conserved C-terminal octapeptide of each A chain (see I.4.2). The existence of hybrid insulin-type I receptors in vivo could also partially account for their overlapping effects (Moxham et al., 1989).

IGF-I and insulin receptors belong to the subclass II family of cell surface glycoproteins (Lammers et al., 1989). Members of this subclass are synthesized as precursors that are processed and oligomerize to yield functional heterotetrameric receptor complexes. A single 1,367 amino acid polypeptide serves as the precursor for each mature receptor (Ullrich et al., 1986). The immature pro-receptor is synthesized as a disulphide linked polypeptide dimer which is subsequently glycosylated,

proteolytically processed into two subunits, α and β, and capped with terminal sugars (Czech et al., 1989). The two extracellular α subunits (~135kda) and two transmembrane β subunits (~90kda) generate the functional β-α-α-β complex after removal of a 30 residue signal peptide (Ullrich et al., 1986). Each receptor spans the plasma membrane via the two β subunits, protruding from the cell surface by 195 amino acids. It is these protrusions to which the entire exofacial α subunits are attached (Corps, 1990; Czech et al., 1989).

The extracelluar ligand binding pockets are formed by the α subunits and possibly some portion of the extracelluar β subunits. There is one high affinity binding site per receptor monomer (McDonald et al., 1989). It is likely that the ligand-receptor interaction principally involves hydrophobic and hydrogen bond contacts leading to conformational changes in both hormone and receptor (induced fit) (McDonald et al., 1989). This distortion leads to transmembrane signal transduction via intramolecular and/or intermolecular allosteric stimulation, culminating in receptor cytoplasmic domain activation. The cytoplasmic domains of IGF type I receptor and insulin receptor include: sequences which confer tyrosine kinase specificity, tyrosine side chain autophosphorylation sites, ATP binding sites and sequences determining intracellular substrate specificity. Upon receptor binding, the ligand stimulates intracellular tyrosine specific kinases leading to β subunit autophosphorylation. Subsequent phosphorylation of cytoplasmic components occurs in a signal specific cascade (Corps, 1990; Czech, 1989; Czech et al., 1989). The similarity between the type I IGF and insulin receptors approaches 65% in

the ligand binding domain, 44% in the α subunit C-terminus and β subunit extracelluar sequences, and 84% in the tyrosine kinase domain (Werner et al., 1989). Despite the overall homology in deduced primary amino acid structure, hydropathy and structural domain distribution, the C-terminal tails of the β subunits contain at least three discrete regions of sequence divergence (Czech et al., 1989; Ullrich et al., 1986). These sequences presumably confer the unique tyrosine kinase interaction that permits specific signal transduction.

Since kinase activation is retained even if the ligand dissociates (Czech, 1989), a critical step in mediating cellular response to IGF is termination of the signal. IGF-receptor complexes cluster in clathrin coated pits, are internalized by endocytosis, and are degraded, at least in part, via the lysosomal pathway (Lammers et al., 1989; Schalch et al., 1986). While the hormone and its receptor are reduced by the cell, any furtherance of the IGF message is facilitated by association of ligand with newly inserted membrane bound receptors.

Notwithstanding the slight differences in peptide sequence and structural composition, type I IGF and insulin receptor cDNAs display differing patterns of hybridization with cellular ribonucleic acid. Rat IGF type I receptor appears to be translated from mRNA species of 11.0 and 7.0kb (Ullrich et al., 1986; Werner et al., 1989) whereas insulin receptor from species reported to be of 10.3, 9.6, 8.5, 7.5, 6.0 and 4.0kb (Briata et al., 1990; Goldstein and Kahn, 1989; Heidenreich et al., 1988). The presence of alternative 3′ UT sequences, in insulin receptor mRNAs, may

account for some of its described transcript size heterogeneity. No mRNA idiosyncrasies are yet characterized to account for transcript size heterogeneity in type I IGF receptors.

The IGF type II receptor (IGF-II receptor) exists as a single transmembrane glycoprotein with almost all of its 250kda mass in the extracelluar domain. Although it can bind IGF-II with a k_d of 0.3nM and IGF-I with a 30 fold lower affinity, it appears that IGF-II exerts its mitogenic effects via the type I receptors (Ballard et al., 1988; Verland and Gammeltoft, 1989). The type II receptors small cytoplasmic portion lacks apparent tyrosine kinase activity (Czech et al., 1989). Bovine type II receptor shows a high sequence homology to the mannose-6-phosphate receptor (man-6-p) which binds sugar moieties associated with several terminal glycoproteins destined for intercellular degradation (Clemmons et al., 1989; Czech, 1989). A hypothesis has been forwarded that IGF-II associates with this receptor not for cellular signalling, but perhaps for processing or elimination (Clemmons, 1989).

Expression of the IGF receptor genes, like the expression of their ligand genes, appear developmentally regulated (Werner et al., 1989). In the myoblast stage of skeletal muscle development, there is a preponderance of IGF type I and II receptors and fewer insulin receptors. The quantity of both IGF receptors decreases upon differentiation with the levels of type I and insulin receptors increasing soon after (Beguinot et al., 1985). IGF-II may regulate its prenatal mitogenic effects primarily through the type II receptor and cease such stimulation when the receptor

number falls. IGF receptor kinases are moreover proposed to be regulated by several intracellular agents i.e. cyclic AMP and phorbol esters (Zorzano et al., 1988). A counter-regulatory relationship may exist between ligand induced phosphorylation effect and the phosphorylation/dephosphorylation activity of these other molecules.

It seems likely that the extent of IGF activity in any tissue will depend on the relative representation of ligand and receptor. Since the intracellular signal reflects the number of occupied receptors, and receptor expression is a dynamic process, the magnitude and spectrum of IGF responsiveness can be influenced dramatically by receptor turnover. High plasma IGF levels appear to precipitate a down regulation of their receptors, concomitant with a cellular desensitization to each ligand. Conversely, the reverse process of up-regulation and hypersensitization as IGF levels decline, also occurs (Blum et al., 1989; Tollefsen et al., 1989a).

I.5 HYPOTHESIS AND OBJECTIVES

Based on the background described, further investigations into the apparent relationship between IGF-I influence and postnatal skeletal muscle growth in cattle were undertaken. The following hypothesis and objectives may be stated:

Hypothesis #1: The number and intensity of apparent IGF-I transcripts on Northern analysis would vary with conditions of hybridization. These conditions include the nature of the radiolabelled probe, the stringency

of the hybridization, and the stringency of the post-hybridization wash.

Hypothesis #2: Differences in amount or species of IGF-I/type I IGF receptor mRNA transcripts in muscles of 3 strains of cattle; PW, SY, and DM, may contribute to the differential growth observed between the 3 strains. Further, that these differences would be apparent during the first 6 months of postnatal development, a period characterized by rapid muscle growth.

Hypothesis #3: Serum concentrations of IGF-I in PW and SY strains are likely to correlate with body weight gain and animal size during the first 6 months of postnatal development.

The primary objectives of this investigation were to:

1.) develop a Northern blot hybridization analysis protocol to validate and reproducibly analyze authentic IGF-I mRNAs in small quantities of bovine skeletal muscle and hepatic tissue.

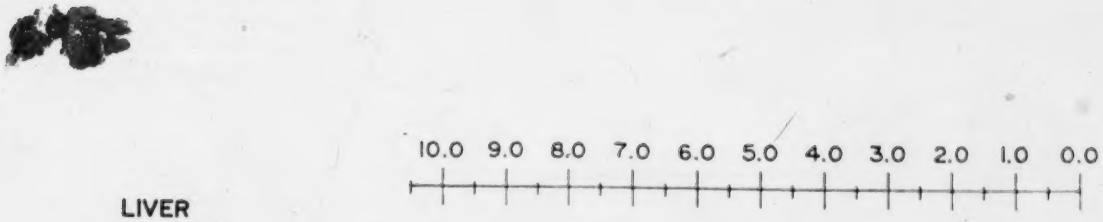
2.) characterize skeletal muscle expression of IGF-I and type I IGF receptor mRNAs by Northern blot hybridization analysis, such that heterogeneity in rate of expression or transcript species throughout a limited period of development, can be examined in relation to possible autocrine/paracrine contributions to the variable muscle growth of PW, SY, and DM cattle.

3.) evaluate tissue and/or species specificity of IGF-I and type I IGF receptor mRNA expression.

4.) describe the developmental profile of circulating IGF-I in PW and

SY cattle strains by radioimmunoassay (RIA), in a manner that would allow the detection of trends or correlations of IGF-I endocrine expression to the variable growth of the same strains.

Figure I.1 Schematic comparison of IGF-I transcripts (recorded in kilobases (kb)) in liver and muscle. Hybridizing mRNAs detected in rat (R) and human (H) tissues by protocols employing random primer probes (RP), end-labelled probes (EL), riboprobes (RB) or nick translated probes (NT).



LIVER

Goldstein et al., 1988
R / NT — 9.0; 1.5; 1.0-0.6

Hoyt et al., 1988
R / RP — 7.5-7.0; 4.7; 1.9-1.5; 1.2-0.9

Hynes et al., 1987
R / NT — 7.5; 4.7; 1.7; 1.2

Lowe et al., 1989
R / RB — 7.5; 1.7; 1.2-0.8

Murphy et al., 1987a
R / NT — 7.0; 3.9; 2.6; 1.8; 1.1-0.7

Murphy et al., 1988
R / NT — 7.0; 3.9; 1.8; 1.0

Roberts et al., 1987b
R / RP — 8.6; 2.1; 1.4-1.0

Han et al., 1988
H / EL — 8.0; 6.3; 0.7

Rotwein, 1986
H / NT — 6.3; 3.7; 1.7; 1.1

Su et al., 1989
H / NT — 7.0; 5.2; 4.7; 2.4; 0.9

MUSCLE

Edwin et al., 1989
R / NT — 8.6

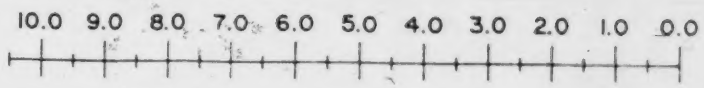
Han et al., 1988
H / EL — 8.0; 6.3

Lowe et al., 1988
R / EL — 7.5; 4.7; 1.7; 1.2-0.8

Murphy et al., 1987a
R / NT — 7.0; 3.9; 2.6; 1.8; 1.1-0.7

Murphy et al., 1987b
R / NT — 7.0; 1.8; 1.1-0.7

Figure I.2 Schematic comparison of IGF-I transcripts (recorded in kilobases (kb)) in multiple tissues. Hybridizing rat (R) and human (H) mRNAs detected by protocols employing random primer probes (RP), end-labelled probes (EL), riboprobes (RB) or nick translated probes (NT).



BRAIN

Lowe et al., 1988
R /EL — 7.5, 4.7, 1.7, 1.2-0.8

Murphy et al., 1987b
R / NT — 7.0, 3.9, 2.6, 1.8, 1.1-0.7

Murphy et al., 1987a
R / NT — 7.0, 1.8, 1.1-0.7

HEART

Murphy et al., 1987b
R / NT — 7.0, 3.9, 2.6, 1.8, 1.1-0.7

Roberts et al., 1987b
R / RP — 8.6, 2.1, 1.4-1.0

PITUITARY

Fagin et al., 1988
R / RP — 8.0, 6.2, 3.8, 2.4, 1.3-1.0

Lowe et al., 1989
R / RB — 7.5, 1.7, 1.2-0.8

MAMMARY

Huff et al., 1986
H / RP — 4.7, 1.4, 0.3

Murphy et al., 1987b
R / NT — 7.0, 1.8, 1.5, 1.1-0.7

KIDNEY

Bell et al., 1985
H / NT — 7.7, 5.3, 0.9

Lowe et al., 1988
R / EL — 7.5, 4.7, 1.7, 1.2-0.8

Murphy et al., 1987b
R / NT — 7.0, 3.9, 2.6, 1.8, 1.1-0.7

TESTIS

Lowe et al., 1988
R / EL — 7.5, 4.7, 1.7, 1.2-0.8

Murphy et al., 1987b
R / NT — 7.0, 3.9, 2.6, 1.8, 1.1-0.7

LUNG

Davenport et al., 1988
R / NT — 7.6, 4.7, 1.7, 1.0

Lund et al., 1986
R / NT — 7.5, 4.7, 1.7, 1.2

Murphy et al., 1987b
R / NT — 7.0, 3.9, 2.6, 1.8, 1.1-0.7

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II. A METHODOLOGICAL VALIDATION OF SPECIFIC INSULIN-LIKE GROWTH FACTOR I (IGF-I) mRNA TRANSCRIPT EXPRESSION

II.1 INTRODUCTION

A typical eukaryotic cell contains ~10^{-5}ng of total ribonucleic acid (RNA) partitioned as approximately 80% ribosomal, 15% small molecular weight species (tRNA, sRNA) and 5% messenger RNA (mRNA) (Maniatis et al., 1982). Probing such a heterogenous population of RNA for specific messages depends on the fractionation of biologically active mRNA, with subsequent exploition of nucleic acid complementarity. The specificity of homologous radiolabelled probe association, or hybridization, is dependant on the hybridization medium, the nature of the probe, and the stringency of post hybridization washes. Criteria influencing specificity in hybridization medium include: temperature, monovalent cation concentration, organic solvent concentration and length of incubation (Albretsen et al., 1988; Church, 1973). Hybridization is normally performed at a temperature some 25°C below the mean thermal denaturation temperature (Tm) of the duplex (Church, 1973). At this temperature, an optimization is attained promoting complementation events (nucleation) between probe and message, while inhibiting mismatched pairing. Factoring into mathematical equations determining the Tm of both DNA/RNA and RNA/RNA hybrids (Albretsen et al., 1988; Biorad, 1989) are cationic influences. These influences affect noncovalent stability between probe and message through modulation of the inherent molecular charge of the polynucleotides. The inclusion of an organic solvant such as formamide also affects probe/message stability by decreasing the predicted Tm by

0.72°C for every 1% increase in solvent concentration (Albretsen et al., 1988). Formamide denatures and hydroxymethylates RNA, reducing hydrogen bonding of any but optimal nucleations (Church, 1973). Through manipulation of these association parameters, and in combination with period of incubation, the rate of both first order (nucleation) and second order (zippering) reactions are affected.

Specificity of association is further influenced by the nature of the probe and its homology to the message. Two commonly generated probes are of a cDNA or cRNA (riboprobe) species. A higher degree of sensitivity is exhibited by riboprobes resulting from their uniform length and the greater inherent stability of RNA/RNA hybrids. However, to establish unambiguous matches, their greater Tm and nonspecific adhesiveness when of length 1kb or greater (Biorad, 1989), must be compensated for by raising hybridization temperatures and/or lowering ionic strength appropriately.

Non-specific binding of labelled probe to membrane-fixed RNA can be reduced post hybridization by a series of stringent washes designed to denature all but the most stable hybrids (Han et al., 1988). Through alteration of salt concentration and wash temperature, conditions close to the Tm of the desired probe/message duplex can be generated.

In a series of experiments, different methodological aspects of the hybridization technique were studied to ascertain the specificity of signals detected between a ^{32}P labelled human IGF-I probe (hIGF-I), and

bovine and rodent cellular RNA. IGF-I is a mitogenic polypeptide mediating growth hormone action (Salmon and Daughaday, 1957), that exhibits structural and functional homology with pro-insulin (Rinderknecht and Humbel, 1978). It exerts pleiotropic effects on diverse cell types through interaction with specific high affinity cell surface receptors and with locally produced binding proteins (Binoux et al., 1986). Northern blot hybridization analysis in multiple tissues has revealed size heterogeneity of IGF-I mRNAs not accounted for by known primary transcript variability (Bell et al., 1986; Fotsis et al., 1990; Rotwein et al., 1986). Multiple IGF-I transcripts are generally considered to be processing variants resulting from as yet undetermined mechanisms. Two clearly defined and strongly hybridizing mRNAs, in the estimated size range of 7.5 - 7.0kb and 1.2 - 0.9kb, have been detected in almost all investigations (Adamo et al., 1988; and Bell et al., 1985; Hoppener et al., 1988). Additional and less well defined hybridizing bands of estimated size 9.0 - 8.0kb (Han et al., 1988; Roberts et al., 1986; Tricolli et al., 1986), 6.2 - 4.7kb (Han et al., 1988; Hoyt et al., 1988; Su et al., 1989), 3.9 - 3.2kb (Murphy et al., 1987; Roberts et al., 1986; Rotwein, 1986), and 2.0 - 1.4kb (Roberts et al., 1987a; Rotwein, 1986; Tricolli et al., 1986) have also been detected. It is possible that the less defined species could be specific to certain tissues or stages of development (Hoyt et al., 1988; Lowe et al., 1989; Roberts et al., 1987b). Alternatively, they may result from nonspecific hybridization of probe to somewhat homologous transcripts, including 28S and 18S ribosomal RNA.

The development of protocols suitable for the specific study of IGF-

I expression in various tissues may help to validate the above described hybridizing signals. Moreover, such protocols will facilitate the investigations of Chapters III and IV.

II.2 EXPERIMENTAL PROTOCOL

One 13 month old Hereford cross steer was killed and skinned according to abatoir standards. The right hepatic lobe and right biceps femoris muscle were removed, dissected into roughly 3 gram portions, placed in preweighed 15 x 75mm polypropylene tubes and immediately frozen in liquid nitrogen. Two six week old male Sprague Dawley rats weighing approximately 100g each were killed by cervical dislocation. The liver and gastrocnemius muscle were excised and stored as described above. Samples from all animals were held at -70°C until RNA extraction. Prior to RNA isolation, tissues were removed from the freezer and pulverized under liquid nitrogen with a mortar and pestle. Powder of approximately 1g dry weight was suspended in 4M guandine isothiocyanate[1] (GIT) and homogenized with a polytron apparatus[2]. This mixture was subsequently layered onto a 5.7M cesium chloride (CsCl) cushion. Total RNA was pelleted by centrifugation in a Sw 41-Ti[3] rotor for 23 hours at 30,000 rpm at 20°C (Chirgwin and Przybyla, 1979). The pellet was solubilized in water, resuspended, and quantified by ultraviolet spectrophotometry at 260nm. Polyadenylated (poly A^+) RNA was fractionated from total RNA by

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[1]All reagents were molecular biology grade or of the highest purity available.

[2]Brinkmann, Westbury, N.Y.

[3]Beckman Instruments Inc., Mississauga, Ontario, Canada.

two cycles of oligodeoxythymidylate cellulose[4] (oligo d(t)) chromatography (Lund et al., 1986) using low ionic strength lithium chloride based buffers (Sambrook et al., 1989).

Total and poly A^+ RNA pellets were dried under vacuum in a Savant speed-vac and brought up to a volume of 5µl with water. To each sample was added 25µl of a buffer containing 30% deionized formamide, 4% glycerol, 10% of 37% formaldehyde, 6% 10x MOPS (0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA), and 3.2% of 0.01% bromophenol blue. The samples were heated at 65°C for 10 minutes then chilled on ice. Ethidium bromide (EtBr) (1µl at 1mg/ml) was added and the sample was microfuged briefly before loading onto a 1% agarose/2.2M formaldehyde gel. RNA was size fractionated, along with a standard RNA ladder[5] (0.24-9.49kb), for 9 hours at 40 volts in recirculating 1x MOPS running buffer. The integrity and accuracy of specimen quantitation were confirmed by examining EtBr staining of ribosomal RNA under brief ultraviolet exposure. The gel was soaked twice for 20 minutes in 10x standard saline citrate (SSC) (1x SSC is 0.15mol NaCL/L, 15mmol trisodium citrate/L, ph 7.0) and blotted to a quaternary amine derived nylon membrane, zeta-probe[6], by the capillary method of Southern (1975). Transferred RNA was fixed to the membrane by baking under vacuum for 1.5 hours at 80°C.

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[4]Type 3; Collaborative Research, Bedford, MA.

[5]Bethesda Research Laboratories, Gaithersburg, MD.

[6]Biorad Laboratories, Richmond, California.

Prehybridization of the membrane occurred for 3 hours at either 50°C or 42°C for riboprobes or random primer labelled cDNA probes respectively. The riboprobe buffer consisted of 60% deionized formamide, 10% dextran sulfate, 1% sodium dodecylsulfate (SDS), 1x SSPE (0.18mol NaCL/L, 10mmol sodium phosphate/L (ph 7.4), 1mmol EDTA/L, 0.5% blotto (low fat milk powder) and 500μg/ml sheared salmon sperm DNA (ss-DNA). Membranes for random primer labelled probes were prehybridized in a buffer containing 50% deionized formamide, 4x SSPE, 1% SDS, 0.5% blotto, 10% dextran sulfate and 500μg/mL ss-DNA. Volumes for prehybridization and hybridization solutions were the same for both probe types (0.25ml/cm^2 of membrane). Hybridization was at either 50°C, 57.5°C, or 60°C for the riboprobe conditions and 43°C for the random primer probe. Hybridization was for 16 hours in fresh prehybridization buffer with the addition of 1.5×10^6 cpm/ml radiolabelled hIGF-IB RNA (Rotwein, 1986), or 3.8×10^8 dpm of random primer labelled hIGF-IB cDNA. Single stranded antisense riboprobes were synthesized from either a Pvu II or Avi II linearized pGEM-1/hIGF-IB plasmid (Rotwein, 1986), using SP6 polymerase[7], [α-^{32}P] CTP (800 ci/mmol)[8], and unlabelled ATP, GTP and UTP. The 1190 nucleotide Pvu II linearized riboprobe consists of 182 bases of 5′ UTR, 585 nucleotides of open reading frame, 369 bases of 3′ UTR and 54 nucleotides of plasmid sequence. Linearizing with Avi II generated a 357 base riboprobe of the full 5′ UTR and 175 nucleotides of open reading frame. Random primer

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[7]Promega, Madison, Wis.

[8]NEN Superconcentrated, NEG-008C.

labelling employed a random prime labelling kit[9], hIGF-IB cDNA (1136bp), and [d-^{32}P] CTP (800ci/mmol)[10] with unlabelled ATP, GTP and UTP. Unincorporated [^{32}P] CTP from all probes was removed from the full length message by sephadex G-50[11] spun column chromatography.

Post hybridization, the riboprobe membranes were rinsed at room temperature in 2x SSC and then washed for 15 minutes in 2x SSC/0.1% SDS. A subsequent wash at 68°C was performed in 0.2x SSC/.1% SDS, 0.1x SSC/0.1% SDS or 0.05x SSC/0.1% SDS for membranes hybridized at 50°C, 57.5°C and 60°C respectively. A final rinse for each blot was at room temperature in 0.2x SSC. The random primer labelled membranes were initially rinsed in 2x SSC/0.1% SDS then washed for 15 minutes in each of: 2x SSC/0.1% SDS at room temperature, 0.5x SSC/0.1% SDS at room temperature and 0.1x SSC/0.1% SDS at room temperature. A final wash in 0.1x SSC/1% SDS was for 30 minutes at 52°C.

Following washing, all blots were air dried, heat sealed in plastic bags and exposed to Kodak XAR 5[12] film at -70°C with Dupont lightening plus intensifying screens. Blots used for sequential probing were stripped of probe by washing twice in 0.1x SSC/0.1% SDS for 20 minutes at 95°C. Stripping effectiveness was confirmed by re-exposure of blots for 24

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[9]Boehringer Mannheim.

[10]New England Nuclear.

[11]DNA grade; Pharmacia Canada.

[12]Eastman Kodak, Rochester, NY.

hours.

II.3 RESULTS

Three different antisense hIGF-IB probes corresponding to two different length riboprobes and one random primer labelled cDNA, were used to analyze mRNA in bovine and rat tissues. A variety of RNA transfer blots were prepared using total RNA and polyadenylated RNA (poly A^+) to ascertain contributions of poly A^+ species to IGF-I transcript levels. Sense strand hybridization of each probe in parallel blots detected no signal (data not shown), supporting the general specificity of signals present in antisense probe hybridizations.

With all probe types, bands of 7.7kb, 4.6kb, and 1.7kb were detected in bovine liver, bovine muscle, and rat liver (Figure II.1 A, B, C, Figure II.2 B). Additional and less well defined bands appeared to be tissue or probe specific. Pvu II linearized riboprobes identified transcripts at 3.5kb across all tissues, and 3.1kb and 2.7kb in bovine liver and muscle (Figure II.1 A). Only bovine liver retained the 3.5kb signal under hybridization with IGF-IB random primer labelled cDNAs or Avi II linearized riboprobes. The Pvu II linearized riboprobe, along with the random primer labelled cDNA further hybridized to a 10.3kb RNA exclusively in bovine liver (Figure II.1 A, C). Both Pvu II and Avi II linearized riboprobes detected a 9.4kb signal only in bovine muscle (Figure II.1 A, B). All 3 probes hybridized to a 1.1-0.8kb species in bovine and rat liver but not bovine muscle (Figure II.1 A, B, C, Figure II.2 B).

The intensity of each signal varied according to tissue and probe type studied. The 10.3kb and 9.4kb RNA of bovine liver and muscle respectively, appeared of consistent intensity across similar quantities of RNA. Neither of these species was readily detectable below 20µg of total RNA (Figure II.1 A). IGF-I signals of 7.7kb decreased progressively in intensity from rat liver to bovine liver to bovine muscle. The strongly hybridizing liver specific transcript of 1.1-0.8kb appeared more intense in bovine than rat liver (Figure II.2 B).

Oligo d(t) chromatography resulted in a marked enhancement of the 10.3kb, 9.4kb, 7.7kb and 1.1-0.8kb transcripts over total RNA in respective tissues (Figure II.1 A, Figure II.2 B). Muscle specific signals were generally only detectable in polyadenylated RNA. A comparison of poly A^+ depleted RNA (A^-) after one and two rounds of oligo d(t) passage respectively, showed a progressive intensity decrease of specific mRNA transcripts, inversely paralleled by an intensity enrichment of these signals in poly A^+ containing elutions (Figure II.2 B).

To further clarify signals of 4.6kb, 3.5kb, 3.1kb, 2.7kb and 1.7kb, several different hybridization and washing parameters were imposed. Elevation of hybridization temperature, and/or stringency of post-hybridization washes, dramatically decreased intensity of 4.6kb, 3.1kb and 2.7kb species (Figure II.1 A, Figure II.2 A,B (intermediate stringency results not shown)). The intensity of the other bands were also affected but to a lesser degree.

II. 4 DISCUSSION

Each of the three hIGF-IB probes employed hybridized with RNA from rat liver, bovine liver, and bovine muscle. With the exception of the 10.3kb band in bovine liver and the 9.4kb band in bovine muscle, characterized IGF-I signals are corroborated by previous analysis in rat (Hoyt et al., 1988; Lowe et al., 1989; Murphy et al., 1987) and human tissues (Han et al., 1988; Su et al., 1989). The 1.1-0.8kb group of transcripts has been detected in muscle by some investigators (Lowe et al., 1989; Murphy et al., 1987) while other studies do not report its presence (Han et al., 1988; Murphy and Friesen, 1987). Fotsis et al (1990) have recently shown that bovine and human IGF-I cDNAs encode identical transcripts; as such, these significant phylogenic differences in mRNA length were unexpected. Bovine species and tissue specific characteristics in IGF-I mRNA expression are reported for the first time in this investigation.

Although most signals in each tissue were clearly defined, hybridization patterns and intensities between probe types varied appreciably. Riboprobes appeared to increase the sensitivity of the hybridization procedure revealing IGF-I messages in bovine liver and muscle that were not detected by random primer probes. Supporting the premise that longer RNA probes are innately more adhesive than shorter probes (Biorad, 1989), the Pvu II linearized riboprobe associated with transcripts in these same tissues that were apparently below the level of detectability for Avi II linearized riboprobes. Owing to the conditions utilized, and the relative low degree of sequence homology between the

hIGF-IB probes and other members of the insulin gene family (<65%, Rotwein, 1986), signals resulting from cross-hybridization with insulin or IGF-II did not seem likely.

Initial hybridization conditions were determined from analysis of thermal denaturation temperatures for each DNA/RNA and RNA/RNA hybrid (Albretsen et al., 1988; Biorad, 1989). With riboprobes, proportional increases in both hybridization temperature and formamide concentration were necessary to retain probe specificity in the face of the intrinsically high lability of RNA. Even so, the apparent sensitivity increase shown by riboprobes was tempered with the realization that RNA probes have a tendency to hybridize to nonspecific transcripts including ribosomal RNA (rRNA). Comparison of hIGF-IB cDNA to human and rat 28S and 18S rRNA sequences (Genebank, IBI, Pustell Sequence Analysis Program), suggests that, at most, 4 complementation foci of less than 10 bases each could be generated. Since 10 bases is generally considered a minimum for nucleation, little reassociation of these RNA probe sequences with rRNA would be expected. However, in the present investigation and other published studies, signals in the vicinity of ribosomal transcripts (4.6kb, 1.7kb) ubiquitously appear. These bands have been reported as authentic IGF-I messages in a number of tissues (Edwin et al., 1989; Goldstein et al., 1988; Huff et al., 1986), employing both radiolabeled RNA and DNA probes (Han et al., 1988; Lowe et al., 1989; Murphy and Friesen, 1988; Roberts et al., 1987b). The abundance of these transcripts have also been shown to increase or decrease under experimental manipulation (Hoyt et al., 1988). However, this finding could be

indicative of parallel but non-specific probe hybridization to variable expression of ribosomal RNA, reflective of cellular protein synthesis.

Comparisons of hybridization patterns in polyadenylated, total, and A^- RNA suggests that bands of 4.6kb and 1.7kb do not represent specific IGF-I mRNA. Postranscriptional polyadenylation of heteronuclear RNA is a common feature in the biogenesis of most eukaryotic mRNA (Jacobson, 1987). Ribosomal and small molecular weight RNA species are not dependent on polyadenylation to fulfil processing requirements or to afford nucleocytoplasmic transport. A single passage of total RNA through columns of oligo (dt) cellulose is proposed to yield an RNA population that is approximately 50% mRNA. Supplementary passage increases message purity to ~90% polyadenylated species (Jacobson, 1987). Hybridizing bands of 10.3kb, 9.4kb, 7.7kb, 3.5kb and 1.1-0.8kb were sequentially enriched over total RNA following 1 and 2 rounds of poly A^+ fractionation respectively. Transcripts of 4.6kb and 1.7kb were not enriched but remained constant in their intensity. In light of the ionic influence that residual CsCL from RNA isolation procedures has on poly A^+ isolation buffers, and of the resultant rRNA aggregation with mRNA specific elutions (Chirgwin and Przybyla, 1979; Krawetz and Anwar, 1984), the authenticity of 4.6kb and 1.7kb signals becomes suspect.

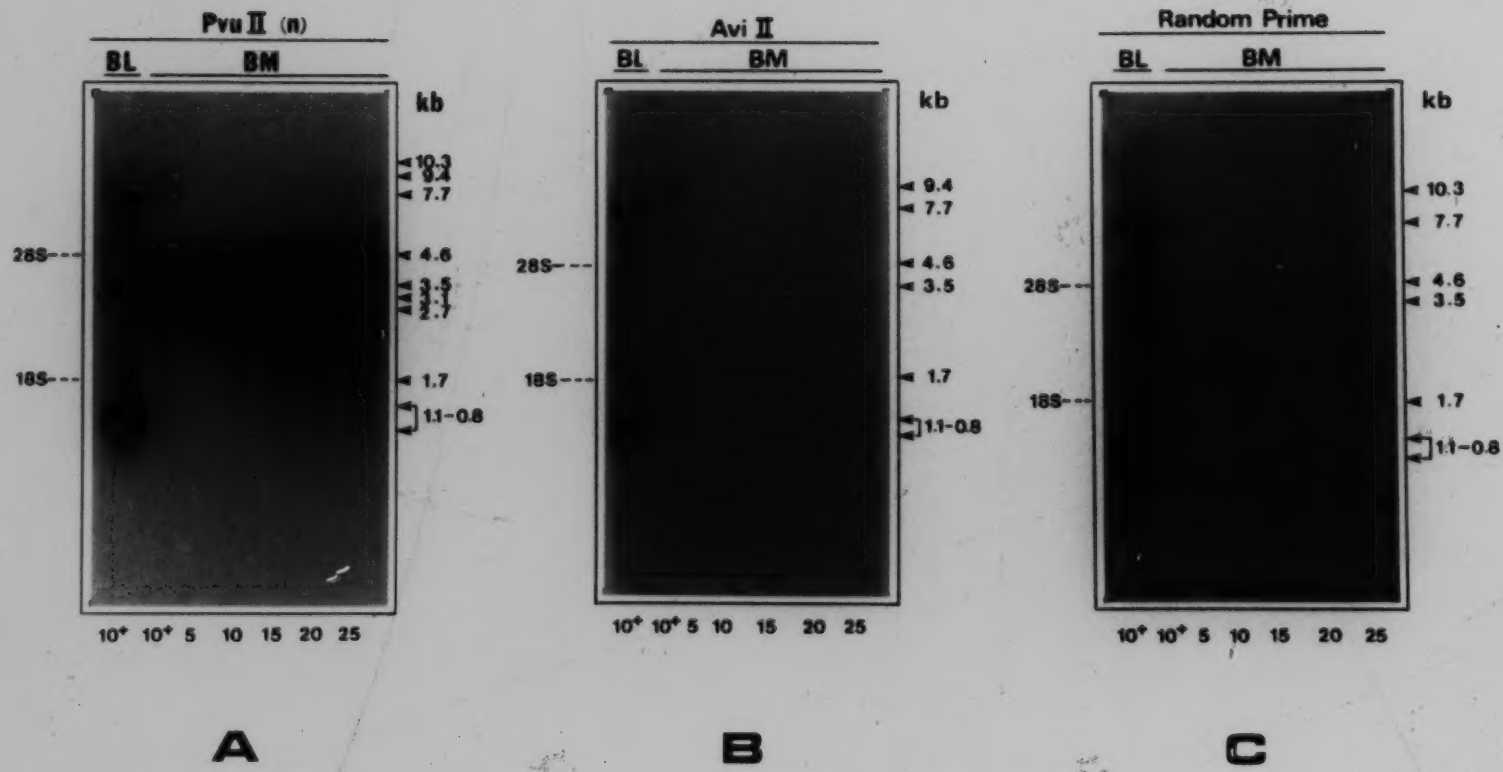
Using what appeared to be the most sensitive probe, the Pvu II linearized riboprobe, an increase in hybridization stringency dramatically reduced signal intensities of all messages in each tissue. Hybridizing bands at 3.1kb, 2.7kb and 1.7kb were erased in bovine liver, while only

bands of 7.7kb and 4.6kb remained in bovine muscle. Rat liver RNA retained signals detected in pilot studies (7.7kb, 4.6kb, 3.5kb, 1.7kb, 1.1-0kb), yet the conspicuousness of these bands was diluted as well. Subjective comparisons incorporating the above described intensity reductions, supported by the minimal detection of certain signals on blots hybridized with random primer labelled cDNA and/or Avi II linearized riboprobes, supports the contention of nonspecificity for 4.6kb and 1.7kb messages. Moreover, transcripts of 3.5kb and 2.7kb in bovine muscle and rat liver, as well as the 2.7kb band in bovine liver, also appeared to result from nonspecific hybridization.

In summary, it is suggested that transcripts of 10.3kb, 7.7kb, 3.5kb and 1.1-0.9kb in bovine liver, 9.4kb and 7.7kb in bovine muscle, and 7.7kb and 1.1-0.8kb in rat liver, appear to represent specific IGF-I mRNA. It is further suggested that to accurately interpret hybridizing signals in Northern analysis, a combination of different probe and hybridization methodologies should be employed. As such, subsequent IGF-I Northern analysis in this thesis will incorporate Pvu II and Avi II linearized hIGF-IB riboprobes, under 'normal' hybridization and wash conditions (described in II.2), in poly A^+ RNA. The high sensitivity of the Pvu II linearized probes, paired with the presumed specificity of the Avi II linearized probes, will permit accurate interpretation of specific IGF-I mRNA.

Figure II.1 Autoradiograms of Northern blots of poly(A^+) and total RNA from bovine liver (BL) and bovine muscle (BM), hybridized with 3 types of [^{32}P] hIGF-IB probe.

As described in the experimental protocol (Section II.2) and represented by autoradiograms A, B and C respectively, the hybridization probes included; a Pvu II linearized hIGF-IB riboprobe, an Avi II linearized hIGF-IB riboprobe and a random primer labelled hIGF-IB cDNA. Hybridization conditions for each probe were those detailed as being normal stringency in Section II.2. Estimated molecular weight (in kilobases (kb)) of the major RNA transcripts, relative to migration of a RNA ladder, are designated to the right of each blot. The position of 28S and 18S ribosomal RNA is indicated to the left. RNA quantity (in μg) is depicted below each lane. Suprascript ($^+$) denotes poly(A^+) RNA enriched by 2 rounds of oligo (dt) chromatography. Autoradiograms shown are 48 hr, 360 hr, and 220 hr exposures of blots A, B and C respectively. Each was exposed at -70°C with intensifying screens.


Pvu II (n)
BL
BM
kb
10.3
9.4
7.7
28S
4.6
3.5
2.7
18S
1.7
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10⁴ 10⁴ 5 10 15 20 25
A
Avi II
BL
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28S
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18S
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1.1-0.8
10⁴ 10⁴ 5 10 15 20 25
B
Random Prime
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kb
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18S
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C

Figure II.2 Autoradiograms of Northern blots of poly(A^+), total and poly(A^-) RNA from bovine liver (BL), bovine muscle (BM) and rat liver (RL), hybridized to a Pvu II linearized [^{32}P] hIGF-IB riboprobe under stringent hybridization conditions (hybridization protocol and probe characteristics are as described in experimental protocol).

Blot A was initially probed with hIGF-IB under normal (n) stringency hybridization conditions (see experimental protocol), stripped (see experimental protocol) and subsequently reprobed under high (h) stringency hybridization conditions. The estimated molecular weight (in kilobases (kb)) of the major RNA transcripts, relative to migration of a RNA ladder, are designated to the right of each blot. The position of 28S and 18S ribosomal RNA is indicated to the left. RNA quantity (in μg) is depicted below each lane. Suprascript ($^+$) denotes poly(A^+) RNA enriched by 2 rounds of oligo (dt) chromatography. Suprascripts (1^-) and (2^-) denote poly(A^-) RNA from one and two rounds respectively of oligo (dt) chromatography. Autoradiograms shown are 192 hr and 140 hr exposures of blots A and B respectively, at -70°C with intensifying screens.

Figure II.2 Autoradiograms of Northern blots of poly(A^+), total and poly(A^-) RNA from bovine liver (BL), bovine muscle (BM) and rat liver (RL), hybridized to a Pvu II linearized [^{32}P] hIGF-IB riboprobe under stringent hybridization conditions (hybridization protocol and probe characteristics are as described in experimental protocol).

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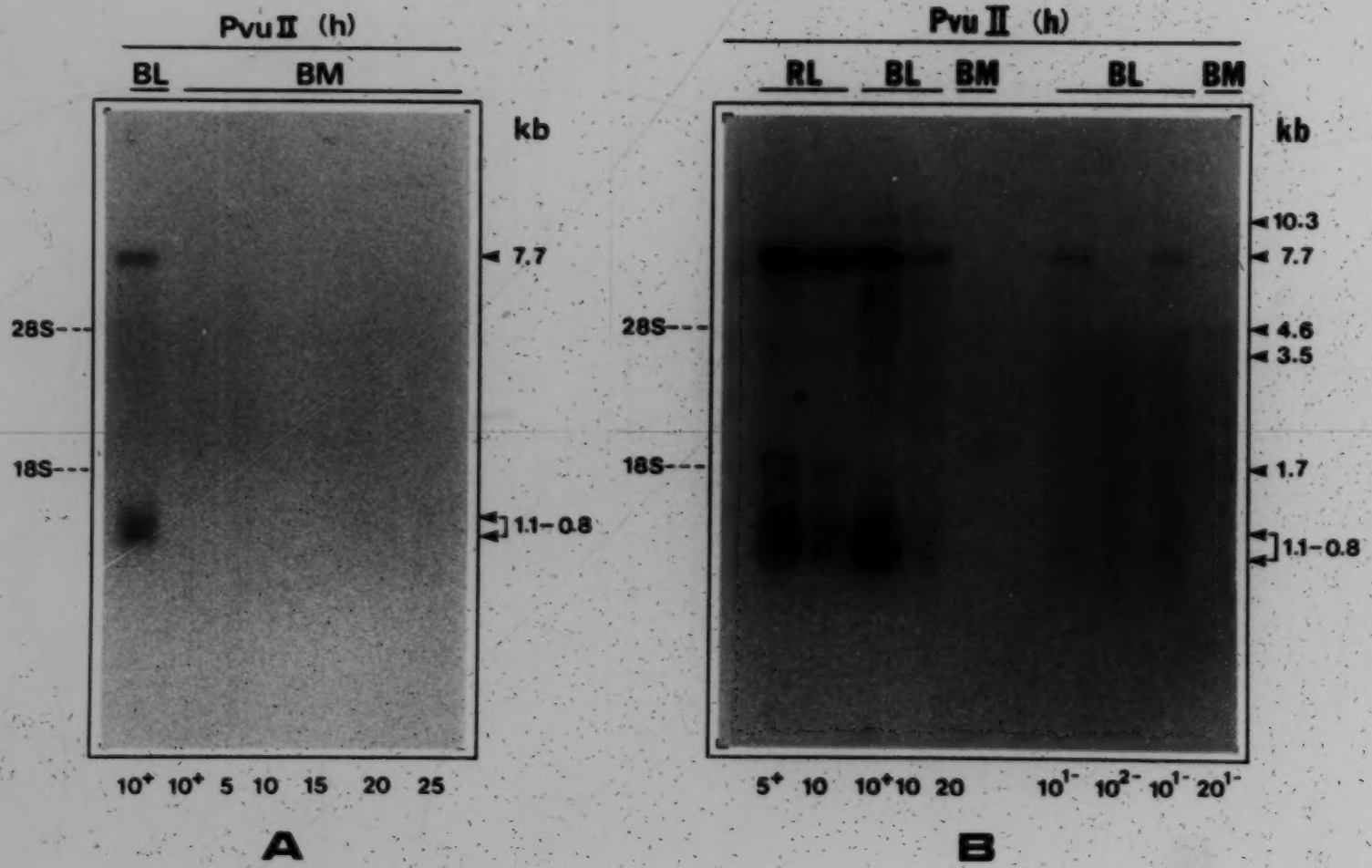
Pvu II (h)
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Pvu II (h)
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5+ 10 10+10 20 10^1- 10^2- 10^1- 20^1-
B

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III. TISSUE AND SPECIES SPECIFICITY OF INSULIN-LIKE GROWTH FACTOR I (IGF-I) AND TYPE I IGF RECEPTOR mRNA

III.1 INTRODUCTION

Insulin-like growth factor I (IGF-I) is a mitogenic polypeptide that functions as the primary mediator of the effects of growth hormone (Roberts et al., 1986; Salmon and Daughaday, 1957). It displays structural and functional homology to pro-insulin (Rinderknecht and Humbel, 1978), exerting pleiotropic effects on diverse cell types through interaction with specific high affinity cell surface receptors (Ballard et al., 1988; Bassas et al., 1987; Burant et al., 1987), and with locally produced binding proteins (Binoux et al., 1986; Poggi et al., 1979). Along with its clearly identified endocrine role (Lowe et al., 1988; Lund et al., 1986), local synthesis of IGF-I has been detected in many tissues suggesting an autocrine or paracrine capacity (D'Ercole et al., 1984; Han et al., 1987a,b; Murphy et al., 1987; Zannger et al., 1986). The observation that different tissues have substantially different complements of messenger RNA sequences, suggests that inter-tissue and inter-species (phylogenic) comparisons of IGF-I and IGF receptor mRNAs may describe peculiarities in these polypeptides' biogenesis.

Evaluation of cDNAs encoding IGF-I has disclosed that the structural and functional domains of the mature growth factor are highly conserved across domestic cattle (Fotsis et al., 1990), man (Rotwein, 1986), rats and mice (Bell et al., 1986; Roberts et al., 1987b). It further predicts that in each species, IGF-I is synthesized as a precursor with extensions

at both amino and carboxyl termini. In domestic cattle and man, carboxyl termini extensions undergo alternative splicing events to generate at least two potential IGF-I translation products. IGF-IA mRNA encode exons 1,2,3 and 5 (153 residues), while IGF-IB contain exons 1,2,3 and 4 (195 residues) (Bell and Merryweather, 1984; Fotsis et al., 1990; Rotwein, 1986). Exons 4 and 5 code for unique E domain terminal peptides whose function, either in relation to the immature growth factor, or as separate entities, is unknown. Reflective of its bovine and human counterparts, the rat IGF-I gene encodes a similar sequence that diverges 16 residues 3′ to the nucleotide initiating the E domain. Inclusion of a 52 base "mini-exon" in the IB transcript leads to a shift in translational reading frame and stop codon compared to IGF-IA (Lowe et al., 1988). Characterized cDNA sequences reveal further heterogeneity in that IA or IB transcripts may contain any of 3 alternative 5′ untranslated regions (UTRs) classified as A,B or C (Roberts et al., 1987a). Heterogeneity of IGF-I transcripts in both rats and humans is appended by the discretionary use of five and three different near consensus polyadenylation signals, respectively (Daughaday and Rotwein, 1989; Fagin et al., 1989; Rotwein, 1986).

Despite the amino acid sequence similarity among IGF-I precursors from different species, very little is known about biosynthesis of IGF-I or about processing steps required to generate the mature transcript. Northern blot hybridization analysis in human and rodent RNA has revealed diverse IGF-I mRNA classes ranging in estimated size from 9.0-7.0kb to 1.0-0.8kb (Adamo et al., 1988; Bell et al., 1985; Hoppener et al., 1988;

Roberts et al., 1987b; Su et al., 1989). As the longest characterized IGF-I cDNA of ~1.15kb (Roberts et al., 1987a) is sufficient to encode pre-pro IGF-I, the size of the smaller mRNAs can be accounted for by the mechanisms outlined in the paragraph above. The sequence and capacity of larger molecular weight mRNA species remains undetermined, although exon specific IGF-I probes hybridize to these transcripts in rat RNAs (Daughaday and Rotwein, 1989). Bovine tissue has yet to be examined in terms of potential autocrine/paracrine expression of IGF-I. Species and/or tissue specific IGF-I requirements may be manifest by variable transcript expression in comparison to human and rat.

As hormone responsiveness is determined by the enterprise of both ligand and receptor in target tissues, IGF-I receptor expression is also important for regulation of IGF-I activity. The human type I IGF receptor is a heterotetrameric protein, synthesized from a single polypeptide chain precursor (280kda) that is glycosylated and cleaved into respective subunits (Ullrich et al., 1986). Northern analysis of tissues exhibiting this receptor commonly report 2 type I transcripts of 11.0kb and 7.0kb (Ullrich et al., 1986; Werner et al., 1989). There is presently no clear information regarding how this mRNA heterogeneity relates to possible tissue or species requirements in the capacity of the receptor protein.

Since each tissue within each species may respond to distinct cis- and/or trans-activation, it is of interest to determine if IGF-I and/or IGF-I receptors are partially regulated by variable genetic expression. In this study, species and tissue specific heterogeneity in the mRNA

expression of both these polypeptides is investigated in domestic cattle and rats.

III.2. EXPERIMENTAL PROTOCOL

Tissues utilized in this analysis were taken from animals as described in Chapter II (II.2). Poly A^+ RNA was isolated by the procedures detailed in the experimental protocol of the same chapter. Bovine pituitary poly A^+ RNA was kindly provided by laboratory co-workers employing identical isolation protocols. Northern transfer blots were initially probed with a Pvu II linearized pGEM-1/hIGF-IB (Rotwein, 1986) antisense riboprobe (characterized in II.2) under low stringency hybridization and wash conditions (described as 'normal' in II.2). The potential for direct comparisons of the results obtained in this study with those from Chapter II, preempted confirmation of signal specificity by parallel hybridization of blots with an Avi II linearized probe. Following autoradiography, membranes were stripped of probe by washing twice in 0.1x SSC/0.1% SDS for twenty minutes at 95°C. Effective stripping was confirmed by re-exposure of blots for 24 hours. Membranes were subsequently reprobed with an Eco RV linearized pGEM-3/human type I IGF receptor riboprobe (Ullrich et al., 1986), under the same hybridization and wash conditions as described above.

III.3. RESULTS

Hepatic and extrahepatic levels of mRNA specific for IGF-I were assessed via hybridization with a Pvu II linearized pGEM-1/hIGF-IB (Rotwein, 1986) riboprobe. As shown in Figure III. 1., increasing amounts

of extracted poly A^+ RNA from rat liver, and bovine liver and muscle, yielded a progressive increase in both common and apparent tissue specific autoradiograph signal intensities. Previous experiments (Chapter II) suggest that not all these signals are specific to IGF-I transcripts. The 4.6kb, 3.1kb, 2.7kb and 1.7kb bands across each tissue, and the 3.5kb band detected in bovine muscle, are proposed to result from nonspecific binding of probe to ambiguous RNA species including 28S and 18S rRNA.

Besides the characteristic 7.7kb and 1.1-0.8kb bands common to both bovine and rat liver, bovine liver exhibited what appeared to be species specific signals at 10.3kb and 3.5kb. Differences were also manifest by IGF-I signal intensity disparity. Spectrophotometric and EtBr staining confirmation of poly A^+ RNA quantity and integrity, permited a realistic comparison of this disparity. At the same total RNA quantity, both the 7.7kb and 1.1-0.8kb rat liver RNAs displayed clearly enhanced hybridization compared to bovine liver. Moreover, the 10.3kb and 3.5kb band exclusive to bovine liver, were both less intense than either the 7.7kb or 1.1-0.8kb signals of bovine or rat liver.

Tissue expression differences of IGF-I mRNA were also revealed within a species (Figure III.2.). In the bovine, mRNA of 9.4kb were detected in muscle and pituitary that were not observed in liver. Conversely, bovine liver displayed a strong signal at 1.1-0.8kb and a less intense band at 3.5kb, neither of which were observed in muscle or pituitary. Further differences were noted in the intensity of the signals both common and specific to muscle, liver, and pituitary. The relative intensity of the

7.7kb mRNA band was greatest in liver followed by muscle and then pituitary. Additionally, the 9.4kb and 7.7kb signals of muscle and pituitary were of similar intensity within each tissue, but both transcripts were less defined in the pituitary.

The subsequent reprobing of blots described by Figure III.1 with an Eco RV linearized pGEM-3/type I IGF receptor (Ullrich et al., 1986) riboprobe, permitted a tissue and species specific comparison of this gene's expression (Figure III.3). Although structural similarities between type I IGF receptor and insulin receptor approach 84% (Ullrich et al., 1986), type I transcripts did not appear to crosshybridize with reported insulin receptor transcripts (Heidenreich et al., 1988). Signals of estimated size 11.0kb, 6.1kb, 4.4kb and 3.1kb were evident across all blots. Notwithstanding the absence of tissue or species specific changes in size distribution of the receptor mRNAs, differences were apparent in hybridization signal intensities between rat liver, bovine liver and bovine muscle. These differences appeared to reflect specific changes in abundance of IGF-I receptor mRNAs, as quantities of RNA probed were similar. The 11.0kb and 6.1kb signals were the strongest and most well defined in bovine muscle, followed by bovine liver and then by rat liver. Furthermore, rat liver mRNA of estimated size 4.4kb and 3.1kb appeared more intense than that detected in bovine muscle or liver respectively.

III.4. DISCUSSION

Examination in this study of extrahepatic distribution of IGF-I mRNA, demonstrates the existence of species and tissue specific variation in

IGF-I transcripts. Although bovine and human IGF-I cDNAs have been shown to encode identical sequences (Fotsis et al., 1990), liver specific phylogenetic mRNA heterogeneity was evident (Han et al., 1988). This is an unexpected result as human and rodent IGF-I, which display 5′ and 3′ disparity in their cDNA (Lowe et al., 1988; Roberts et al., 1987a; Rotwein, 1986), hybridize to identical mRNA signals (Han et al., 1988; Murphy and Friesen, 1988). Within a species, hepatic and extrahepatic differences in IGF-I transcript expression were also detected. It can be postulated that production of these mitogenic factors by multiple tissue may reflect a localized control of body growth in which autocrine and paracrine agents augment an endocrine homeostasis. IGF-I transcript abundance or processing alternatives may generate diversity in the capacity of translated proteins contributing to species and/or tissue specific requirements.

IGF-I transcripts were detected in rat liver that are substantiated by published data (Hoyt et al., 1988; Hynes et al., 1987). Bovine liver exhibited a similar, although less intense signal pattern, with the addition of a species specific transcript at 10.3kb. Bovine muscle and pituitary RNA displayed an apparently ubiquitous 7.7kb band and a tissue specific 9.4kb IGF-I message; however, the hepatic assemblage of 1.1-0.8kb transcripts was not evident. The absence of 1.1-0.8kb transcripts in muscle is supported by previous research in man (Han et al., 1988) and rats (Edwin et al., 1989).

Studies indicate that size heterogeneity in mammalian and rodent IGF-

I mRNAs does not simply reflect identified structural differences in 5′ coding sequences or 3′ carboxyl termini (Hoyt et al., 1988). Nor does it appear to represent multiple but discrete copies of the IGF-I gene in nuclear chromatin (Tricoli et al., 1984). The variation in transcript size of IGF-I mRNA, combined with the conserved nature of the mature growth factor, suggests that a considerable portion of the 10.3kb, 9.4kb, 7.7kb and 3.5kb mRNAs may describe diverse and extensive 5′ and 3′ UTRs.

Eukaryotic promoters driving the transcription of heteronuclear RNA (hnRNA) are recognized not by polymerase themselves, but by a host of cis- and trans-active factors which direct RNA polymerase II to the proper 5′ transcription initiation sites. Speculating as to the mechanisms regulating the origin of large molecular weight IGF-I transcripts, and by analogy with IGF-II (Holthuizen et al., 1990), a coordination of tissue specific and developmental dependent factors may be responsible for initiating transcription of multiple mRNA species with divergent 5′ ends. Large 5′ UTRs may be specific for IGF-I polypeptide synthesis regulated by translational efficiency or attenuation. Alternatively, as in the yeast invertase system, two different sites of transcription initiation could be used to generate secreted or cytoplasmic forms of a common mature protein (Carlson and Botstein, 1982; Perlman et al., 1984). Such a mechanism may foster inter-and/or extra-cellular activity for autocrine specific IGF-I (See I.4.2).

Untranslated regions at the 3′ end of mRNA transcripts may be functionally important to the capacity of expressed messages. Such

sequences, inclusive of polyadenylated tracts, could be differentially recognized by species or tissue specific factors affecting nucleocytoplasmic RNA transport, mRNA stability and/or mRNA translatability. Variation in either the length or location of polyadenylation is proposed to account for the size dispersion of 1.1-0.8kb transcripts (Roberts et al., 1987b).

Post-transcriptional processing events such as splicing (consensus and/or silent) and RNA editing (Eisen, 1988; Nevins, 1983), could determine complex transcriptional units responsible for additional diversity and specificity in IGF-I mRNA. Alternative RNA splicing protocols can not only be used to generate related mRNA species from alternative promoters (Schibler and Sierra, 1987), but can also be used to create alternative messages containing different coding sequences (Brietbart et al., 1987). The distinct carboxyl extensions of bovine IGF-IA and IGF-IB precursors reside on separate exons (Fotsis et al., 1990). It is suggested that the combined actions (both synergistic and antagonistic) of multiple tissue or species specific splicing factors, may participate in the synthesis of particular IGF-I precursors (exon specific Northern analysis will be necessary to confirm or discredit this suggestion). Moreover, the extension peptides themselves may subserve discrete functions. RNA editing may also contribute to IGF-I mRNA transcript heterogeneity. Although not yet characterized in higher eukaryotic cells, limited non-template coded 5′ and 3′ uridine additions (Eisen, 1988), or trans-splicing could facilitate species or tissue specific augmentation of IGF-I messages to endow an, as of yet,

undetermined capacity.

Type I IGF receptor mRNA expression also appears to be both tissue and species specific. There is no evidence for size distribution variation in transcripts; however, differences in hybridizing signal intensities are apparent. The two largest type I IGF receptor transcripts have been previously detected in human placenta (Ullrich et al., 1986), mouse fibroblast and mouse adipocyte RNA (Yee et al., 1989). An 11.0kb transcript has also been noted in rat liver (Ullrich et al., 1986) and muscle (Werner et al., 1989). IGF-I receptor mRNAs identified in human placenta are derived from a single gene (Ullrich et al., 1986). It is unknown whether both mRNA code for the same receptor precursor sequence or whether structural variants exist. Recently, Yee et al. (1989) have identified two different rat type I IGF receptor transcripts that vary in the regions encoding the β subunit. Both of these appear to be processing variants of the primary 11.0kb message. Transcript variants of type I IGF receptor β subunits may provide a means for subtle differences in the translated proteins signalling competence in certain tissues or species. The β subunits contain tyrosine-specific protein kinase domains, ATP binding sites and several phospho-tyrosine autophosphorylation acceptor sites (Corps, 1990; Czech et al., 1989), the combination of which mediates IGF-I intracellular signalling.

Since the liver appears to be the major source for circulating IGF-I (Lowe et al., 1988), and high levels of IGF-I precipitate a down regulation of type I IGF receptor (Blum et al., 1989; Tollefsen et al.,

1989), it was not surprising to detect diversity in the intensity of transcript expression between hepatic and extrahepatic tissue. An unexpected observation was that these differences did not appear to represent simple signal intensity differences across all transcripts, but that specific transcripts were affected in specific tissues. Moreover, phylogenetic differences were also observed in type I receptor signal intensities within a tissue. As analysis of RNA by radiolabeled probe hybridization is, by nature, an analysis of steady state, signal intensities represent a static level of transcript. An increase in transcript levels may result from an increased rate of transcription, an increase in the stability of transcripts, or a combination of both.

It is possible that IGF-I receptor transcript length and/or intensity differences may result from alternative promoter/enhancer regulatory regions whose activation or strength of activation is dependent on species or tissue specific factors. Each transcript could encompass variable 5′ and/or 3′ heterogeneity, in turn affecting its translational capacity or its stability. Alternatively, each receptor transcript could theoretically encode independent but related receptor polypeptides manifesting tissue specific responsiveness to ligand. Recent studies in cultured myoblasts (Beguinot et al., 1988) have suggested that phosphorylation of β subunits in specific type I IGF receptors, can result from insulin stimulated tyrosine kinase activation of adjacent insulin receptors. Certain transcripts may encode particular IGF-I receptor polypeptides whose kinases may be able to be activated through this indirect cross-phosphorylation. Other studies (Moxham et al., 1989) have

presented data to suggest that there exists a species of IGF-I receptor that is a hybrid composed of an insulin receptor α-β heterodimer and a type I IGF receptor α-β heterodimer. The function and tissue distribution of these asymetric heterotetramers remains undetermined. However, it is conceivable that specific transcripts could be translated into receptor polypeptides mediating this type of phenomenon in specific cell types.

Type I IGF receptor heterogeneity is also demonstrated by the high affinity ligand binding domain of the α subunit. In the chick, variable carbohydrate moiety extensions are acquired by the α subunit dependent on the tissue of expression (Bassas et al., 1987). Biochemical analysis will be required to define any interrelationship between receptor mRNA transcript expression and/or species variability, and possible specific augmentation of the translated receptor by diverse carbohydrate moieties.

Data obtained from this investigation imply that IGF-I and type I IGF receptor mRNA are variably expressed in different tissues and in different species. Whether the multiple transcripts identified are generated by the combined actions of a variety of cis- and trans-activators, and whether each message can contribute independently to IGF-I responsiveness in certain tissues, remains undetermined. Further studies of IGF-I and type I IGF receptor cDNA, mRNA and genomic DNA sequences, are necessary to define the molecular basis of this transcript heterogeneity.

Figure III.1 Northern blot analysis of IGF-I mRNAs from rat liver (RL), bovine liver (BL) and bovine muscle (BM) poly(A^+) and total RNA, hybridized with a Pvu II linearized [^{32}P] hIGF-IB riboprobe (hybridization conditions were as described in experimental protocol).

Molecular weight (in kilobases (kb)) of the major RNA transcripts, relative to migration of a RNA ladder, are designated to the right of the blot. The position of 28S and 18S ribosomal RNA is indicated to the left. RNA quantity (in μg) is depicted below each lane. Suprascript ($^+$) denotes poly(A^+) RNA isolated after 2 rounds of oligo (dt) chromatography. Autoradiograms shown are 120 hr exposures of RL and BL and 240 hr exposure of BM, at -70°C with intensifying screens.

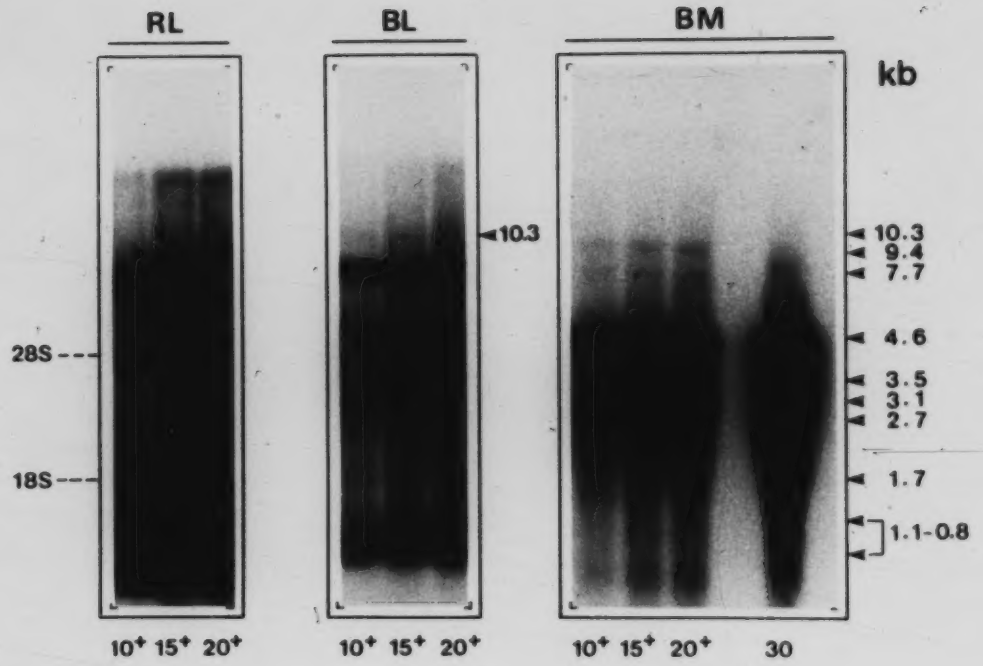

RL
BL
BM
kb
28S
18S
10.3
10.3
9.4
7.7
4.6
3.5
3.1
2.7
1.7
1.1-0.8
10+ 15+ 20+
10+ 15+ 20+
10+ 15+ 20+ 30

Figure III.2 Northern blot analysis of IGF-I mRNAs from rat liver (RL), bovine liver (BL), bovine muscle (BM) and bovine pituitary (BP) poly(A^+) RNA, hybridized with a Pvu II linearized [^{32}P] hIGF-IB riboprobe (hybridization conditions were as described in experimental protocol).

Molecular weight (in kilobases (kb)) of the major RNA transcripts, relative to migration of a RNA ladder, are designated to the right of the blot. The position of 28S and 18S ribosomal RNA is indicated on the left. RNA quantity (in μg) is depicted below each lane. Suprascript ($^+$) denotes poly(A^+) RNA isolated after 2 rounds of oligo (dt) chromatography. Autoradiogram is a 216 hr exposure at -70°C with intensifying screens.

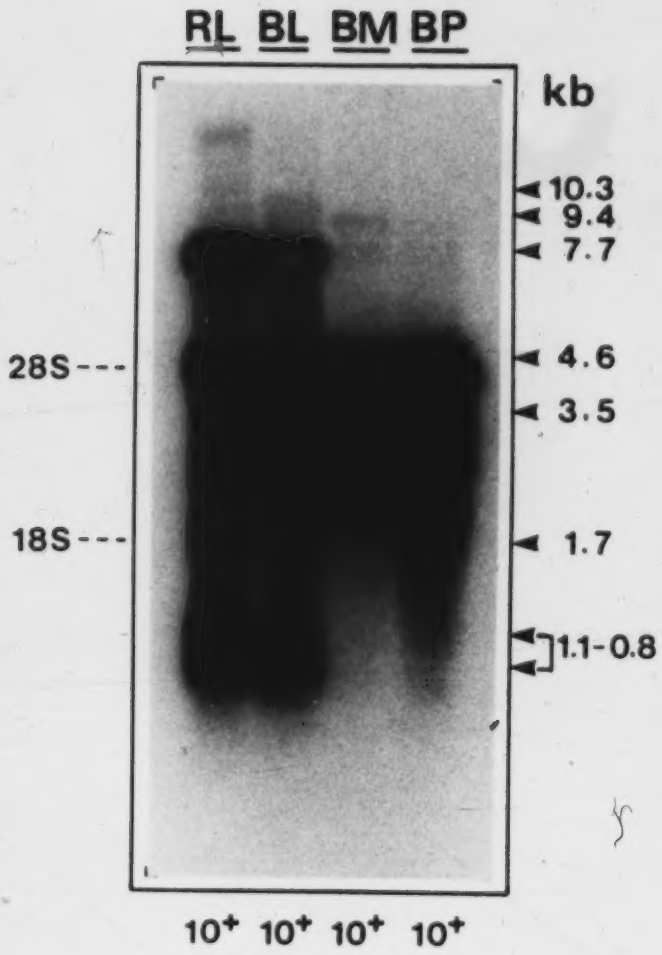
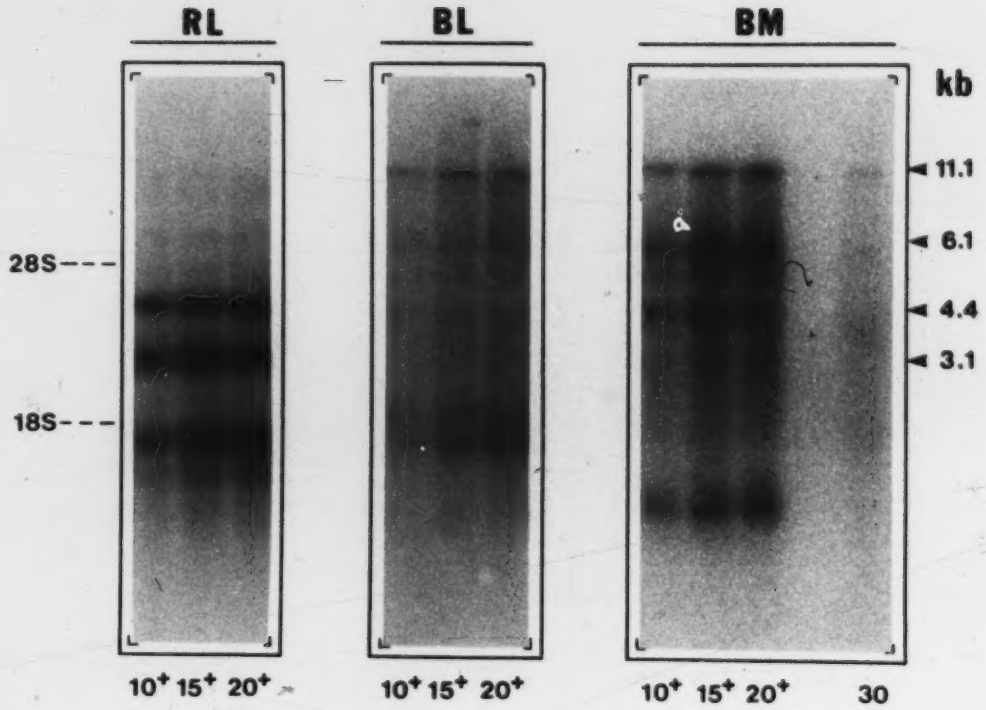

RL BL BM BP
kb
10.3
9.4
7.7
28S---
4.6
3.5
18S---
1.7
1.1-0.8
10+ 10+ 10+ 10+

Figure III.3 Northern blot analysis of type I IGF receptor mRNAs from rat liver (RL), bovine liver (BL) and bovine muscle (BM) poly(A^+) and total RNA, hybridized with an Eco RV linearized [^{32}P] human type I IGF receptor riboprobe (hybridization conditions were as described in experimental protocol).

The blots represented by these autoradiograms were initially probed with hIGF-IB riboprobes (Plate III.1), stripped (as described in experimental protocol) and reprobed with type I IGF receptor riboprobes. Molecular weight (in kilobases (kb)) of the major RNA transcripts, relative to migration of a RNA ladder, are designated to the right of the blot. The position of 28S and 18S ribosomal RNA is indicated on the left. RNA quantity (in μg) is depicted below each lane. Suprascript ($^+$) denotes poly(A^+) RNA isolated after 2 rounds of oligo (dt) chromatography. Autoradiograms shown are 240 hr exposures of RL and BL and a 192 hr exposure of BM, at -70°C with intensifying screens.


RL
BL
BM
kb
11.1
6.1
4.4
3.1
28S
18S
10+ 15+ 20+
10+ 15+ 20+
10+ 15+ 20+ 30

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IV. EXPRESSION OF INSULIN-LIKE GROWTH FACTOR I (IGF-I) AND TYPE I IGF RECEPTOR mRNA IN BOVINE SKELETAL MUSCLE.

IV.1 INTRODUCTION

Insulin-like growth factor I (IGF-I), the putative second message hormone for somatotropic action (Salmon and Daughaday, 1957), is proposed to function as one of the most efficacious stimulators of postnatal body growth (Daughaday, 1984; Underwood and D'Ercole, 1984). Serum IGF-I levels show a positive correlation with birth weight and growth in domestic cattle (Lund-Larsen et al., 1977; Rumsey and Elasser, 1987), man (Furlanetto and Cara, 1986; Merimee et al., 1982), dogs (Eigenmann et al., 1984 a,b) and swine (Buonomo et al., 1987). Murphy et al. (1987b), have further described a strong interdependence linking hepatic and extrahepatic tissue weight with tissue specific expression of IGF-I. In skeletal muscle, IGF-I enhances both proliferation and differentiation (Florini et al., 1986; Jennische and Olivecrona, 1987b; Schmid et al., 1983), promotes glucose and amino acid uptake (Poggi et al., 1979; Zapf et al., 1981) and suppresses protein breakdown (Beguinot et al., 1985; Ewton et al., 1987; Florini, 1987). These growth promoting effects appear to be initiated by ligand binding to specific tyrosine-kinase glycoprotein receptors (Burant et al., 1987; Ewton et al., 1987; Kiess et al., 1987; Shimizu et al., 1986).

Although the strong correlation between hepatic IGF-I content and circulating IGF-I levels supports the premise that liver is the predominant source of this growth factor (Goldstein et al., 1988;

Schwander et al., 1983), multiple extrahepatic tissues, including muscle, have been shown to express IGF-I immunoreactivity (Clemmons and Van Wyk, 1985; D'Ercole et al., 1984; Jennische and Hanson, 1987; Jennische et al., 1987) and mRNA (Edwall et al., 1989; Tollefsen et al., 1989). Studies such as these suggest an autocrine and/or paracrine activity for this growth factor in muscle tissue. Reinforcing this hypothesis, it has been demonstrated that endogenous IGF-I expression is increased during myogenic differentiation (Tollefsen et al., 1989), in regenerating traumatized skeletal muscle cells (Jennische et al., 1987) and, under the influence of GH, in muscle undergoing hypertrophic growth (Isgaard et al., 1988a,b). It is tempting to postulate that the production of IGF-I may reflect a localized control of growth in which its activities may be dependent on tissue and/or developmental requirements.

Variation in the construct of the growth factor itself may further modulate IGF-I activity contributing to specific requirements. Recent analysis of cDNAs encoding IGF-I, in domestic cattle and man, has revealed that IGF-I is synthesized as a precursor with peptide extensions at both the amino and carboxyl termini. The primary IGF-I transcript can undergo alternative splicing events to generate at least 2 potential translation products. IGF-IA mRNA encode exons 1,2,3 and 5 (153 residues), while IGF-IB contain exons 1,2, 3 and 4 (195 residues) (Fotsis et al., 1990; Rotwein, 1986). Exons 4 and 5 code for unique terminal E domain peptides which are cleaved during processing of the pre-pro growth factor (Humbel, 1984). The function of these E peptides, either in relation to the initial translation product, or as separate entities after cleavage, is

unknown.

Northern blot hybridization analysis reveals further IGF-I complexity, identifying heterogenous groups of IGF-I mRNAs ranging in estimated size from 9.3-7.0kb to 1.1-0.7kb (Edwall et al., 1989; Lowe et al., 1988; Murphy et al., 1987b). As the largest characterized IGF-I cDNA of ~1.15kb (Roberts et al., 1987) is sufficient to encode the mature growth factor, the substantial amount of unknown sequence information within the larger molecular weight IGF-I mRNA proves enigmatic in its design.

This investigation sought to elucidate whether IGF-I and/or type I IGF receptor mRNA are differentially expressed in skeletal muscle of three closely related, yet variably muscled, strains of cattle. Moreover, this study attempted to characterize a developmental pattern in the expression of these genes over the first six months of postnatal growth. Differences in selection criteria used for the three breed groups (growth rate for pee-wee (PW) and beef synthetic (SY), expression of inherited muscular hypertrophy for double-muscled (DM)), are hypothesized to have contributed to the variable growth rate of these three strains. Currently, PW are ~1/2 the yearling body weight of SY, while SY display a lower muscle to bone ratio, and a lower proportion of muscle as a percentage of body weight, than do DM (Shahin and Berg, 1985; Makarechian et al., 1984) (for animal description see I.2).

IV.2 EXPERIMENTAL PROTOCOL

Animals employed in this investigation were from the PW, SY, and DM herds maintained at the University of Alberta beef research ranch, Kinsella, Alberta. Five age-matched males from each line, born and raised as commercial beef stock, served as the study group. Skeletal muscle biopsies were procured from each animal at 1, 2, 3 and 6 months of age. Samples were taken from the biceps femoris muscle approximately 20cm distal to the point of insertion, at a depth of ~2cm. This hind quarter musculature was analyzed because of its: ease of access for biopsy, *high-average growth impetus* (Butterfield et al., 1966), and adequate postnatal morphological and biochemical differentiation (Eppenberger et al., 1983). Each animal received a systemic anaesthetic, Rompun (0.05cc/50kg), followed by local anaesthetic (2.0ml of 2% Lidocaine) subcutaneously at the site of biopsy. The incision area was aseptically prepared, hide and fascial severance surgically produced, and a 1 - 2g portion of the muscle tissue was removed utilizing blunt dissection. Post-surgical trauma was minimized by terminating hemorrhage, mattress suturing both fascia and hide, and providing topical and systemic antibiotics. Sequential biopsies were excised from contralateral muscle groups. In circumstances where more than one biopsy was procured from the same limb, the second site was situated approximately 5cm distal to the first.

Tissue samples were placed in preweighed 15 x 75mm polypropylene tubes, rapidly frozen in liquid nitrogen, and stored at -70°C until use. Poly A^+ RNA was isolated by the procedures detailed in the experimental protocol of Chapter II (II.2). Northern transfer blots were probed with

Pvu II and Avi II linearized pGEM-1/hIGF-IB (Rotwein, 1986) antisense riboprobes (characterized in II.2), under low stringency hybridization and wash conditions (described as 'normal' in II.2). Because bovine IGF-I encodes identical cDNA to human IGF-I (Fotsis et al., 1990), the hIGF-I probe was considered suitable for studies in bovine tissue.

Following autoradiograph development, the membranes were stripped of probe by washing twice in 0.1xSSC/0.1%SDS for 20 minutes at 95°C. Effective stripping was confirmed by re-exposure of the blots for 24 hours. Membranes were subsequently reprobed with an Eco RV linearized pGEM-3/human type I IGF receptor antisense riboprobe (Ullrich et al., 1986), under the same hybridization and wash conditions as described above.

IV.3 RESULTS

A Pvu II and an Avi II linearized hIGF-IB riboprobe, together with a type I IGF receptor riboprobe, were used to analyze bovine skeletal muscle mRNAs from PW, SY, and DM animals during the first 6 months of postnatal development. Comparisons of hybridizing IGF-I transcripts among the 3 breed groups provided no evidence for differences in size distribution or signal intensities in the expression of this growth factor at any age. However, there appeared to be an age-related increase in signal intensities over the investigative period. Both IGF-I riboprobes hybridized to similar transcripts in each tissue, yet the apparent superior signal to noise ratio of the Avi II linearized hIGF-IB probed blot provided the best autoradiograph for interpretation. Hybridizing

transcripts of 9.4kb and 7.7kb were detected in all muscle poly A^+ RNAs (Figure IV.1). Additional and less well defined bands of 4.6kb and 1.7kb were also noted. Densitometric scanning analysis of band intensity increases was frustrated due to the faintness exhibited by all signals, even after 20 days of autoradiographic exposure. To provide a characterized reference, poly A^+ RNA from adult bovine and rat livers was analyzed in parallel with the muscle RNA. Liver RNA from both species hybridized to the IGF-I probe in the estimated size range of 7.7kb, 4.6kb, 1.7kb and 1.1-0.8kb. Bovine liver further exhibited bands at 10.3kb and 3.5kb (Figure IV.1, 10.3kb not indicated). These transcripts are corroborated by previous research in domestic cattle (Chapter II), rats (Hoyt et al., 1988; Hynes et al., 1987) and man (Su et al., 1989). Hybridizing band differences between bovine and rodent livers suggests alternative transcript expression, transcript processing and/or transcript stability amongst species (See Chapter III). Tissue specific differences in IGF-I transcripts were evident by the presence of the 9.4kb band and the absence of the 10.3kb and 1.1-0.8kb bands in bovine muscle compared with bovine liver.

Interpreting the authenticity of several of the transcripts identified was problematic due to apparent non-specific hybridization of the riboprobes to RNA species including 28S and 18S ribosomal RNA (Chapter II). Comparison of hybridizing IGF-I species identified in this investigation with those detected in Chapter II, suggests that IGF-I signals of 4.6kb and 1.7kb in bovine liver, rat liver, and bovine muscle, and 3.5kb in rat liver, are non-specific.

Reprobing of the stripped Avi II linearized hIGF-IB probed blot, with a human type I IGF receptor antisense riboprobe, revealed that similar to its primary ligand, type I IGF receptor mRNA signals in skeletal muscle display no size distribution or signal intensity differences among the 3 breed groups at any age. However, an age-related progressive increase in transcript intensity over the experimental period was noted. Bands at 11.0kb, 6.4kb, 4.4kb and 3.1kb were detected in all muscle poly A^+ RNAs (Figure IV.2). These signals are corroborated by previous research (See Chapter III) and are believed to represent specific receptor transcripts. Of interest was the observation that intensity and definition of muscle IGF-I receptor transcripts appeared more enhanced across all animal strains and time periods than did the IGF-I signals. This phenomenon was evident even at significantly disparate autoradiographic exposures (Figure IV.1, IV.2). The small quantity of bovine and rodent liver poly A^+ RNA probed, combined with a relatively short period of autoradiographic exposure, hindered the detection of type I IGF receptor transcripts in these tissues.

IV.4 DISCUSSION

To test the possibility that transcript expression and/or processing heterogeneity in IGF-I and/or type I IGF receptor mRNAs could contribute to the differential muscle growth exhibited between PW, SY and DM cattle, this investigation analyzed muscle poly A^+ RNA by Northern hybridization analysis with riboprobes corresponding to the antisense RNA sequence of each polypeptide. The data presented in this study demonstrates that species of IGF-I and type I IGF receptor transcripts do not differ amongst

the three strains, nor do they differ over the first 6 months of postnatal development. However, the abundance of all messages, as measured by autoradiograph band intensity, progressively increased during the investigative period. These findings suggest that the mRNA for IGF-I and type I IGF receptor may be developmentally regulated at the level of gene expression and/or mRNA stability, and further suggests that both these polypeptides may be important for postnatal skeletal muscle growth.

It has been previously demonstrated that there is an age-related increase in serum concentration of IGF-I in the bovine post-partum (Lund-Larsen et al., 1977); it is inferred from rodent investigations that this increase is attributed to IGF-I derived from hepatic tissue (D'Ercole et al., 1984; Hoyt et al., 1988). It appears that simultaneous with this developmentally related endocrine increase, extrahepatic expression of IGF-I may also be elevated. The signals that modulate this expression increase in skeletal muscle of PW, SY, and DM cattle are unknown. The best characterized regulator of IGF-I is growth hormone, which rapidly increases IGF-I transcription in muscle and many other tissues (Hynes et al., 1987; Lowe et al., 1987; Murphy et al., 1987a). Insulin (Clemmons and Shaw, 1983) and non-hormonal factors such as cell density and cell cycle timing (Clemmons and Van Wyk, 1985) also regulate secretion of this mitogen. Further investigations will be required to define which, if any of the aforementioned modulators, is responsible for the age-related increase in skeletal muscle IGF-I expression during development. Additionally, longer term studies are needed to precisely characterize the temporal parameters of this progressive increase.

As in hepatic tissue (Han et al., 1988; Hynes et al., 1987; Su et al., 1989), skeletal muscle IGF-I transcripts appear heterogenous in size exhibiting signals at 9.3kb and 7.7kb. These hybridizing species are corroborated by earlier research in Hereford bull calves (See Chapter II). Inferring from the observation that pre-pro IGF-I appears to be encoded by a single gene in humans and rodents (Rotwein et al., 1986), bovine IGF-I message heterogeneity is likely to represent either initiation or processing variants of a primary transcript. Since the large molecular weight IGF-I species do not appear to undergo maturation dependent shortening (Lowe et al., 1989), and since an IGF-I cDNA of ~1.15kb is sufficient to encode the mature growth factor (Roberts et al., 1987), the capacity of these large messages remains undetermined. Based on the observation that the most 3′ of characterized polyadenylation sites lies within 1kb of the growth factor coding region (Shimatsu and Rotwein, 1987), and that no 5′ cap site for IGF-I has yet been identified, it is likely that transcripts greater than ~2.0kb encode large 5′ untranslated regions (UTRs). By analogy with IGF-II (Holthuizen et al., 1990), it is conceivable that developmental and/or tissue specific promoters may drive the transcription of IGF-I from variable upstream locations, thereby generating 5′ UTRs of differing length. This mechanism could help to explain the differences noted between the largest molecular weight IGF-I species in bovine liver and bovine muscle (Figure IV.1). In turn, these long 5′ UTRs may affect the translational capacity of messages, allowing for translational as well as transcriptional control of IGF-I gene expression in specific tissues.

In addition to possible 5′ contributions to IGF-I message heterogeneity, bovine IGF-I has been shown to differ in its 3′ exons and in the length of its 3′ UTRs (Fotsis et al., 1990). The different 3′ sequences described by both IGF-IA and IGF-IB (See Section IV.1) could influence translatability or stability of transcripts (Jackson and Standart, 1990). The construction of the probes used in this study were such to detect simultaneously both IGF-IA and IGF-IB. Hence, the contributions of known 3′ divergence to bovine IGF-I transcript heterogeneity could not be assessed. Exon specific probe investigations will be required to elucidate whether PW, DM or SY skeletal muscle selectively express IGF-IA or IGF-IB or both.

Although IGF-I transcript expression and/or species heterogeneity does not appear to directly contribute to the variable muscle growth of PW, DM and SY animals, tissue specific IGF-I expression may still be linked to differential growth but only in concert with other independent factors whose expression may be species and/or developmentally regulated (Leof et al., 1982). Moreover, an additional complexity in determining the actions of IGF-I in many tissues is the presence of IGF binding proteins (BP). Binding proteins have been shown to both enhance (Elgin et al., 1987) and inhibit (Rutanen et al., 1988) the actions of IGF-I. Studies of these proteins will be required to clarify their role in muscle growth and development.

IGF-I responsiveness can be considered to be partially determined by the coordinate modulation of both ligand concentration and receptor

expression in target tissues. It is evident from this investigation that IGF-I receptor expression is a dynamic characteristic of bovine skeletal muscle. Steady state levels of type I IGF receptor mRNA showed a significant and progressive increase in abundance from birth to 6 months of age. Hybridizing transcripts of 11.0kb, 6.1kb, 4.4kb and 3.1kb were equally affected and displayed no obvious expression or species differences between PW, SY, and DM skeletal muscle. Solution hybridization analysis of the expression of IGF-I receptor in rat skeletal muscle during development (Alexandrides et al., 1989), challenges the progressive type I IGF receptor expression observed in bovine skeletal muscle. In rat muscle, there is evidenced an overall decrease in IGF-I receptor mRNA during the first 30 days of postnatal life. However, concomitant to this IGF-I receptor decrease, insulin receptor expression appears to increase. A relatively high degree of sequence homology between the IGF-I and insulin receptors (Ullrich et al., 1986) permits a cross-reactivity with each others primary lingands. Accordingly, overlapping bioeffects (Rechler and Nissley, 1985; Steel-Perkins et al., 1988), may account for the age-related differences in IGF-I receptor transcript expression observed between bovine and rat skeletal muscle.

The absence of heterogeneity in type I IGF receptor mRNA signal intensities or size distribution between the three strains of cattle, suggests that these factors do not directly contribute to the disparate muscling phenotypes. However, the potential influence of possible strain specific receptor glycosylation (Bassas et al., 1989) and/or intracellular receptor signal modification (Roth and Beaudoin, 1987; Takayama et al.,

1984) remain important questions for future study.

Although the biological significance and the translational competence of the multiple IGF-I and type I IGF receptor mRNA transcripts remains uncertain, the results of this investigation are in keeping with the hypothesis that bovine skeletal muscle is a site of IGF-I and type I IGF receptor gene expression. It is conceivable that the mRNA expression level differences observed in the present study, represent a mechanism to effect cell responsiveness (albeit the rate of synthesis of both these polypeptides may also be altered at the translational level independent of mRNA accumulation). An understanding of the molecular mechanisms responsible for the ontogenic patterns will require characterization of the pertinent genetic regulatory regions, chromatin structural modifications and elucidation of the associated trans- acting factors that control expression of the IGF-I and type I IGF receptor genes. Additional studies of the relative stability and translatability of skeletal muscle IGF-I and IGF receptor mRNA species, and of the protein products derived from these mRNAs, could assist in further elucidating their relationship to the differential muscling phenotypes evident between PW, SY, and DM strains of domestic cattle.

Figure IV.1 Autoradiogram of a Northern blot of poly (A^+) RNA from bovine skeletal muscle at different stages in postnatal development, and rat and bovine liver, hybridized with a [^{32}P] hIGF-IB riboprobe (hybridization conditions were as described in experimental protocol).

Hybridizing mRNAs detected in poly (A^+) RNA from skeletal muscle of pee-wee (PW), beef synthetic (SY) and double-muscled (DM) bull calves at postnatal age 1 month (Mo.1), 2 months (Mo.2), 3 months (Mo.3) and 6 months (Mo.6), and hepatic tissue of adult rat (RL) and prepubscent bovine (BL) livers. The amount of poly (A^+) RNA (in μg) is indicated at the bottom of the figure with a suprascript + denoting polyadenylation enhancement. All samples represent RNA from the tissue of 5 animals pooled at the time of RNA extraction. Molecular weight of hybridizing mRNAs at the right of the figure are in kilobases (kb) and are based on comparison with RNA molecular weight markers applied to the blots. The 28S and 18S markers (on left of figure) correspond to the position of the ribosomal RNA bands on the ethidium bromide stained gel. Autoradiogram shown is a blot exposure to Kodak XAR-5 film for 360 hrs at -70°C with intensifying screens.

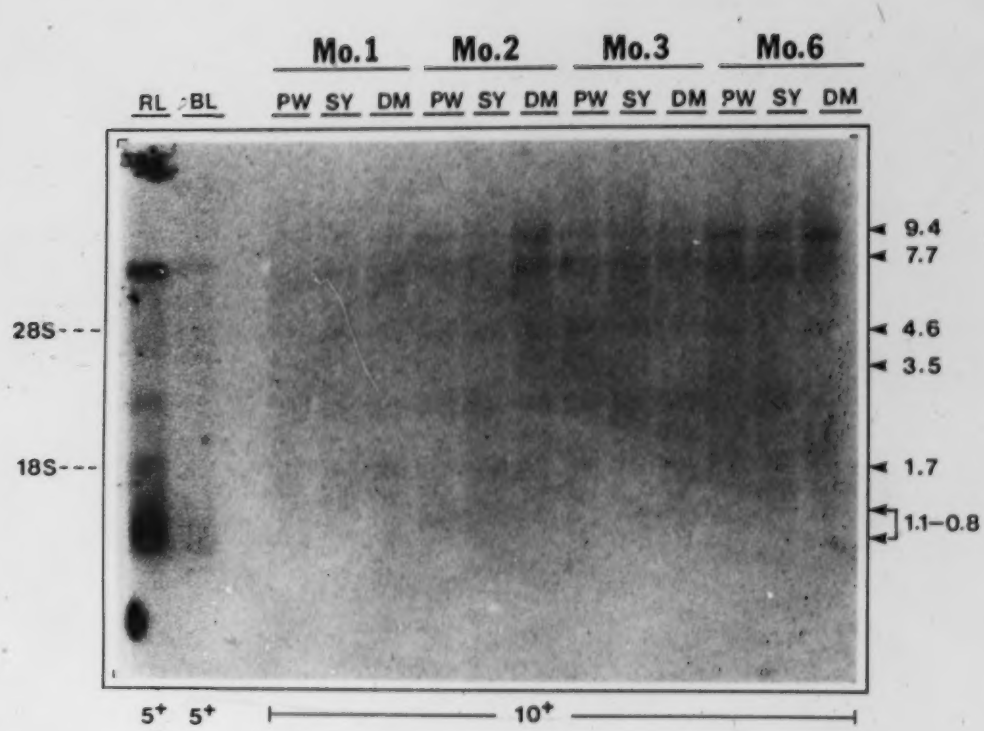
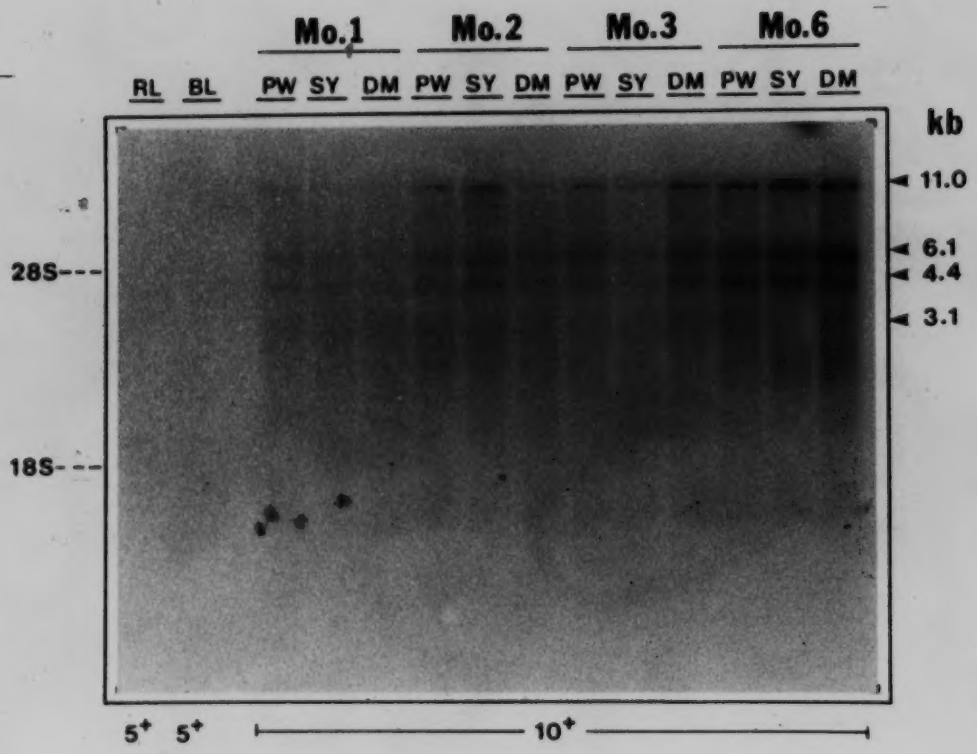

Mo.1
Mo.2
Mo.3
Mo.6
RL
BL
PW SY DM PW SY DM PW SY DM PW SY DM
28S
18S
9.4
7.7
4.6
3.5
1.7
1.1–0.8
5+ 5+
10+

Figure IV.2 Autoradiogram of a Northern blot of poly (A^+) RNA from bovine skeletal muscle at different stages in postnatal development, and rat and bovine liver, hybridized with a [^{32}P] human type I IGF receptor riboprobe (hybridization conditions were as described in experimental protocol).

Hybridizing mRNAs detected in poly (A^+) RNA from skeletal muscle of pee-wee (PW), beef synthetic (SY) and double-muscled (DM) bull calves at postnatal age 1 month (Mo.1), 2 months (Mo.2), 3 months (Mo.3) and 6 months (Mo.6), and hepatic tissue of adult rat (RL) and prepubscent bovine (BL) livers. The amount of poly (A^+) RNA (in μg) is indicated at the bottom of the figure with a suprascript + denoting polyadenylation enhancement. All samples represent RNA from the tissue of 5 animals pooled at the time of RNA extraction. Molecular weight of hybridizing mRNAs at the right of the figure are in kilobases (kb) and are based on comparison with RNA molecular weight markers applied to the blots. The 28S and 18S markers (on left of figure) correspond to the position of the ribosomal RNA bands on the ethidium bromide stained gel. This autoradiogram represents the same blot as in Plate III.1, stripped of its [^{32}P] hIGF-IB probe (as described in experimental protocol) and reprobed with a [^{32}P] human type I IGF receptor riboprobe. Autoradiogram shown is a blot exposure to Kodak XAR-5 film for 260 hrs at -70°C with intensifying screens.


Mo.1
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V. DEVELOPMENTAL PROFILES OF INSULIN-LIKE GROWTH FACTOR I (IGF-I) PLASMA LEVELS IN TWO UNIQUE STRAINS OF CATTLE SELECTED FOR MATURE BODY SIZE

V.1 INTRODUCTION

The regulation of postnatal growth and development is a complex process involving an interplay of genetic and environmental factors. A considerable amount of research in domestic livestock has been directed towards the genetic components of this interplay, attempting to elucidate and capitalize on determinants contributing to variability in either the efficiency or magnitude of production. Recently, the capacity of somatotropic agents, such as insulin-like growth factor I (IGF-I), have become an important focus.

IGF-I is a circulating polypeptide that has been shown to be the principal mediator through which growth hormone (GH) exerts its growth promoting effects (Froesch et al., 1985; Schoenle et al., 1982; Schwander et al., 1983). It exerts mitogenic and differentiative actions on a variety of cell types including muscle (Ewton and Florini, 1980; Schmid et al., 1983) and skeletal tissue (Isgaard et al., 1988). Studies have demonstrated that IGF-I circulates in the vasculature non-covalently complexed to specific binding proteins that influence both its physical half life (T 1/2) and its biological actions (Elgin et al., 1987; Hintz, 1984; Rutanen et al., 1988). These BPs disassociate from the ligand prior to IGF-I interaction with specific high affinity cell surface receptors (Czech et al., 1989). Most, if not all characterized IGF-I enterprise is

mediated by the type I IGF receptor, a tyrosine kinase glycoprotein (Ullrich et al., 1986) whose kinase activity is proposed to function in intracellular signalling of the IGF-I message (Beguinot et al., 1985; Ewton et al., 1987).

A substantial body of evidence has accumulated to show that circulating levels of IGF-I are often correlated with growth. In man, serum concentrations are age and sex dependent and show a positive correlation with birth weight (Bala et al., 1981). Individual differences in height and growth velocity of preadolescent children also appear to be related to IGF-I secretory capacity (Gourmelen et al., 1986). Eigenmann et al. (1984a) have observed that levels of IGF-I in 3 distinct breeds of poodles, which differ in growth rate and mature body weight, were highest in the largest (standard) animals and lowest in the smallest (toy). A similar correlation has been noted in macro-, mini-and micro pigs (Buonomo et al., 1987). Moreover, substantial responses in 6 week and mature body weights of mice is manifest after 7 generations of selection for, or against, plasma levels of IGF-I (Blair et al., 1988).

Although there appears to be little relation between body size and circulating IGF-I levels across species (Zangger et al., 1987), the above described data provide strong evidence that within a given species, IGF-I concentrations may well influence the size of individual animals provided a comparable environment and suitable nutrition. As such, producers may be able to use plasma levels of IGF-I as a potential means to rank livestock for selection purposes, attempting to improve either the

efficiency or magnitude of lean meat production. The aims of this investigation were to: 1.) correlate circulating IGF-I concentrations with body weight in domestic cattle belonging to 2 strains that differ in mature size and growth rate, and 2.) characterize circulating IGF-I concentrations within these strains over the first 6 months of postnatal growth.

V.2 EXPERIMENTAL PROTOCOL

Animals used in this investigation were from the pee-wee (PW) and beef synthetic (SY) herds maintained at the University of Alberta beef research ranch, Kinsella, Alberta. Five age matched bull calves from each strain, born and raised as commercial beef stock, served as the study group. The phenotypic differences exhibited between the two strains are the result of many generations of selective breeding for growth rate. Currently, PW yearling body weights are ~1/2 those of SY (for detailed description see I.2)..

At each of the sampling periods, corresponding to 1, 2, 3, and 6 months of age, animals were weighed and 40ml blood samples were procured via jugular venipuncture. Blood was collected into heparinized vacutainers[13] and immediately chilled on ice. Within two hours of collection, the samples were centrifuged at 1,500 x G for 10 minutes at room temperature, and the plasma was subsequently stored in polypropylene tubes at -20°C until analysis. IGF-I secretions do not appear episodic in

[13]Becton Dickinson and Company, Rutherford, NJ.

cattle (Ronge et al., 1988); therefore, the IGF-I status can be accurately described by measuring single daily blood samples.

Measurement of IGF-I and assessment of its activity are complicated by the observation that ~99% of circulating IGF-I is non-covalently complexed to large molecular weight binding proteins (BP) (Clemmons et al., 1989). The affinity of these proteins interferes with detection of IGF-I in plasma or serum by radioimmunoassay (RIA) through competition with antibody for the antigenic site of the growth factor and/or for labelled tracer. In this study, plasma IGF-I was separated from its BP by acid gel filtration (Zapf et al., 1981) with suggested modifications (Daughaday et al., 1986). Plasma IGF-I levels were determined by RIA employing an antibody raised by Underwood and Van Wyk[14] that has a high affinity for IGF-I, displaying only 0.5% cross-reactivity with IGF-II.

Triplicates of each plasma sample were aliquoted to borosilicate assay tubes and desicated under vacuum in a Savant speed-vac. Samples were resuspended in 100µl of assay buffer containing 0.05% Tween 20 (as a substitute for bovine serum albumin), generating an IGF-I dilution of 1:600. In a single assay, all PW and SY samples, together with synthetic standards of IGF-I[15] (0.1 - 3.0ng/ml), were preincubated with antiserum for

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[14]University of North Carolina, N.C., and distributed by the National Hormone and Pituitary program of the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

[15]Bachem Fine Chemicals, Torrence, California. Lot number 588C.

24 hours at 4°C. Iodinated [^{125}I][16] recombinant IGF-I (prepared as in Zapf et al., 1981), of approximately 10,000 cpm/100μl, was added to the assay tubes and allowed to incubate for 24 hours at 4°C. Subsequent addition of 100μl goat antirabbit gamma globulin[17] (diluted 1:25 in assay buffer), and 100μl of normal rabbit serum (diluted 1:150 in assay buffer), separated antibody-bound and free [^{125}I] IGF-I. This mixture was incubated at 4°C for 24 hours, followed by centrifugation (3000 x g, 15 minutes, 4°C), aspiration of supernatant and counting of the precipitate by gamma counter. Intra-assay coefficient of variation (as determined by duplicate controls) averaged 8.2%.

The IGF-I and weight data acumulated were subjected to statistical analysis (SAS, 1987) using weight and/or age and/or IGF-I concentration as the main effects (procedures described in V.3).

V.3 RESULTS

Table V.1 shows the mean weight of the PW and SY experimental animals from birth to 6 months of age. Over the entire investigative period, body weight across strains was significantly different ($p=0.01$). Student-Newman-Keuls (SNK) multiple range test at each sampling period, showed that significant differences occurred at 1, 2, 3 and 6 months of age ($p<0.05$), but not at birth. Repeated measures analysis of variance with weight at each period as the repeated measure, and birthweight, strain and

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[16]Bachem Fine Chemicals, Torrence, California. Lot number ZE426.

[17]Gibco Canada Inc., Burlington, Ontario.

their interaction as independent variables, demonstrated that only the effect of birthweight was significant ($p=0.04$). Average daily gain (ADG) (kg/day) for SY differed significantly from PW ($p=0.03$) (Table V.1).

Circulating IGF-I concentrations were in the range of 164-327ng/ml for SY bull calves and 194-453ng/ml for PW bull calves. Unfortunately, individual samples collected at the third month of age were pooled by accident. As such, for statistical comparisons of IGF-I levels at this period, only the mean and SEM from triplicate analysis of month 3 pooled samples could be used. Repeated measures analysis or variance for mean IGF-I concentration across all sampling periods, demonstrated that plasma IGF-I levels were significantly higher in PW compared with SY ($p=0.02$). Student's T-tests for the same data at each period, showed a significant difference at the second month of age ($p<0.0005$). Both month 1 and 3 showed an indication of difference, but not at significant levels ($p<0.05$) (Figure V.1).

Plotting individual and average IGF-I values (ng/ml) with body weight (kg) for the PW and SY experimental animals, revealed similar downward facing parabolic profiles. However, the PW curves demonstrated a sharper rise, a higher peak and a more pronounced decline (Figure V.2). The absence of individual animal data from the third month, precluded a statistical comparison of these developmental profiles. To determine if body weight was related to IGF-I concentration, simple linear regressions were done at each of the sampling periods. A significant relationship was found to exist only in SY calves at 1 month ($p=0.05$) and 2 months

(p=0.0050) of age. Significance is indicated by regression lines drawn through the appropriate data (Figure V.3).

V.4 DISCUSSION

This present investigation compared body weight at 1, 2, 3, and 6 months of age, and average daily rate of gain over this same period, in PW and SY bull calves. Moreover, circulating IGF-I concentrations were correlated with body weight and time, within and across the two strains.

Although PW calves were significantly lighter (except at birth), and had a significantly lower ADG than SY, hormonal data demonstrated that PW calves had a significantly higher mean circulating IGF-I concentration than did SY over the first 6 months of development. High levels of IGF-I in the PW were unexpected since IGF-I is considered to be the principle mediator of the growth promoting activities of GH (Roeden and Hossner, 1986). Anderson et al. (1988) observed a significant and positive correlation with IGF-I levels and protein deposition in prepubertal steers. As stated earlier, Eigenmann et al. (1984a) and Buonomo et al. (1987) demonstrated a highly significant linear correlation between circulating IGF-I and body size in poodles and swine respectively. It is apparent from this present investigation that these trends don't appear to hold true across PW and SY strains of cattle. It would be of interest for future investigations to determine if the characterized dependence of circulating IGF-I levels on circulating GH levels (Eigenmann et al., 1984b), also does not hold true in PW and/or SY strains.

In each of the PW and SY experimental animals, circulating IGF-I levels increase from 1 month of age to a peak at 2 - 3 months of age and subsequently decline. When graphed, these parabolic profiles, although in keeping with the ontogenic IGF-I expression patterns observed in other mammals (Gluckman and Butler, 1983; Hall and Sara, 1984), display a specific chronological component. IGF-I concentrations in man are low at birth, remain low over the first year of development and then rise gradually through childhood increasing markedly at puberty. The pubertal peak is followed by a progressive decline in IGF-I to average adult levels, attained early in the third decade of life (Hall et al., 1984) In sheep, circulating IGF-I levels are relatively low at birth, rise to a peak at ~40 days of age and subsequently decline to adult levels (Gluckman and Butler, 1983). Although the design behind the patterns remains undetermined, it is possible that species specific requirements are responsible for generating the divergent developmental trends in IGF-I expression apparent between man, sheep, and PW and SY cattle.

Over the six month experimental period, not only was it evident that IGF-I levels did not show a positive linear correlation with weight across PW and SY strains, but it was evident that such correlations were absent within strains as well. The relationships between circulating IGF-I levels and body weight were best described as downward facing parabolas (Figure V.2). Ronge and Blum, (1989) have studied IGF-I expression in Simmental-cross steers between the ages of 15 days and puberty. Their results demonstrate an age dependent increase in IGF-I associated with body mass. However, their experiment omitted analysis at months 2 and 3,

a period shown by this investigation to be manifest by a substantial rise followed by a subsequent decline in plasma IGF-I levels. It is possible that the cattle employed by Ronge and Blum, (1989) would have shown a comparable plasma IGF-I level peak if analyzed at these age intervals. Additionally, IGF-I levels in PW and SY strains may progressively increase from the sixth month of age, paralleling the positive linear correlation with weight observed in the Simmental-cross steers. Longer term plasma hormone studies in PW and SY animals are needed to help confirm this second postulate.

Even though positive correlations between IGF-I levels and body weight were apparently lacking in the PW and SY animals over the entire experimental period, analysis of the SY animals at 1 and 2 months of age demonstrated significant ($p<0.05$) positive linear regressions (Figure V.3). These observations comply with what was initially hypothesized by this investigation, and are corroborated by previous analysis in domestic cattle (Lund-Larsen et al., 1977), man (Furlanetto and Cara, 1986) and dogs (Eigenmann et al., 1984a,b). The fact that similar relationships between IGF-I levels and body weight were not observed at month 6 in the SY, nor at any interval in PW, suggests that this genetic relationship may not be as important in these circumstances for normal postnatal development.

Recent investigations have demonstrated that besides the hepatically derived endocrine IGF-I (Schwander et al., 1983), multiple tissues express IGF-I immunoreactivity (Clemmons and Van Wyk, 1985; D'Ercole et al., 1984)

and mRNA (Edwall et al., 1989; Lund et al., 1986) which may function in autocrine and/or paracrine capacities. As such, although not reflected in plasma values of IGF-I, body weight may positively correlate with tissue levels of this growth factor. There may exist divergence in tissue IGF-I concentrations which could more directly influence the growth process than circulating IGF-I. Earlier investigations (Chapter IV) demonstrated a gradual increase in IGF-I mRNA expression in skeletal muscle of PW and SY animals between 1 and 6 months of age. This same study showed a parallel increase in the expression of IGF-I receptor mRNA. Although these expression increases do not necessarily imply either an autocrine/paracrine possibility in all tissues, or an enhancement of receptor density on all cell surfaces, they do provide for a preliminary interpretation of the developmental relationship between circulating IGF-I and body weight in PW and SY bull calves.

The same reasoning employed above cannot be applied to the relationships observed between IGF-I levels and body weight across the two strains, since transcript levels of both polypeptides in skeletal muscle appeared similar in PW and SY animals. As such, it is suggested that modulation of cellular responsiveness to IGF-I could be as important in regulating growth, as are variations in the expression level (autocrine, paracrine and/or endocrine) of this growth factor. There exists a number of potential mechanisms by which cellular sensitivity to IGF-I can be regulated including: 1.) variation in the affinity of ligand for cell surface receptors (by analogy with IGF-II (Burgissen et al., 1991)), 2.) disassociation of receptor occupancy and effect (e.g. truncated and/or

functionally incompetent receptors), 3.) post-binding effector modulation of signal (e.g. an intracellular decrease in the concentration of second messengers (Nishizuka, 1988), or an increase in the activity of inhibitory agents such as phorbol esters (Zorzanna et al., 1988), 4.) synergistic and/or antagonistic effects between IGF-I and other hormones, growth factors or inhibitors (Bajaj et al., 1982; Leof et al., 1982; Vassilopoulou-Sellin et al., 1984), or 5.) variation in quantity, affinity or activity of IGF-I binding proteins (BPs).

Since it appears that IGF-I is stored as a circulating conjugate complexed to specific binding proteins, potential variability in the capacity of these proteins is a relevant aspect to consider when speculating as to the observed disconnection between circulating IGF-I levels and bodyweight across, and within, PW and SY animals. The physiological functions of circulating IGF-I BPs remain unclear. Present evidence suggests that conjugates do not bind to IGF-I receptors. However, enhancement (Elgin et al., 1987) as well as inhibition (Rutanen et al., 1988) of IGF-I activity have been attributed to BPs. They may modulate the actions of IGF-I in vivo by increasing the growth factors half life and/or changing its accessibility to membrane bound receptors (Ruoslahti and Pierschbacker, 1987). Relevant to this discussion is the recognition that many cell types and tissues secrete BPs (McCusker and Clemmons, 1988; Scott et al., 1985). Accordingly, variability in their synthesis and/or secretion may play a role in the regulation of IGF-I action at the target cell level. Detailed ontogenic studies on the role of the IGF-I binding proteins in modulating IGF-I action in both PW and

SY animals, are required to determine if BPs contribute to the relationships between IGF-I and weight that are observed in these strains over the first 6 months of postnatal life.

The preliminary data accumulated by this study, suggest that circulating IGF-I concentrations may not necessarily be a physiologic indicator of growth potential in PW and SY cattle. As revealed herein, IGF-I concentrations need not be positively correlated with body size and growth rate over the first six months of development. Additional longer term investigations will be required to determine if a positive linear relationship is established after 6 months of age.

TABLE V.1. LEAST SQUARE MEANS OF GROWTH PARAMETERS FOR BEEF SYNTHETIC (SY) AND PEE-WEE (PW) BULL CALVES.

	SY	PW	SE	Probability
No. of animals	5	5		
Birth wt., kg	36.0	28.0	2.06	0.09
1 month wt., kg	74.8	53.6	4.98	0.01
2 month wt., kg	102.6	76.0	6.45	0.01
3 month wt., kg	136.6	104.4	7.93	0.02
6 month wt., kg	238.0	184.6	11.92	0.02
ADG, kg/day	1.12	0.87	0.06	0.03

Figure V.1. Mean plasma levels of insulin-like growth factor I (IGF-I) at 1, 2, 3 and 6 months of age for beef synthetic (SY) and pee-wee (PW) bull calves. Bars are SEM. Student's T-test evaluations of circulating IGF-I values between SY and PW demonstrates a significant difference (at $p=0.05$) only at month 2 ($p=0.004$) (month 1, $p=0.13$; month 3, $p=0.05$; month 6, $p=0.52$).

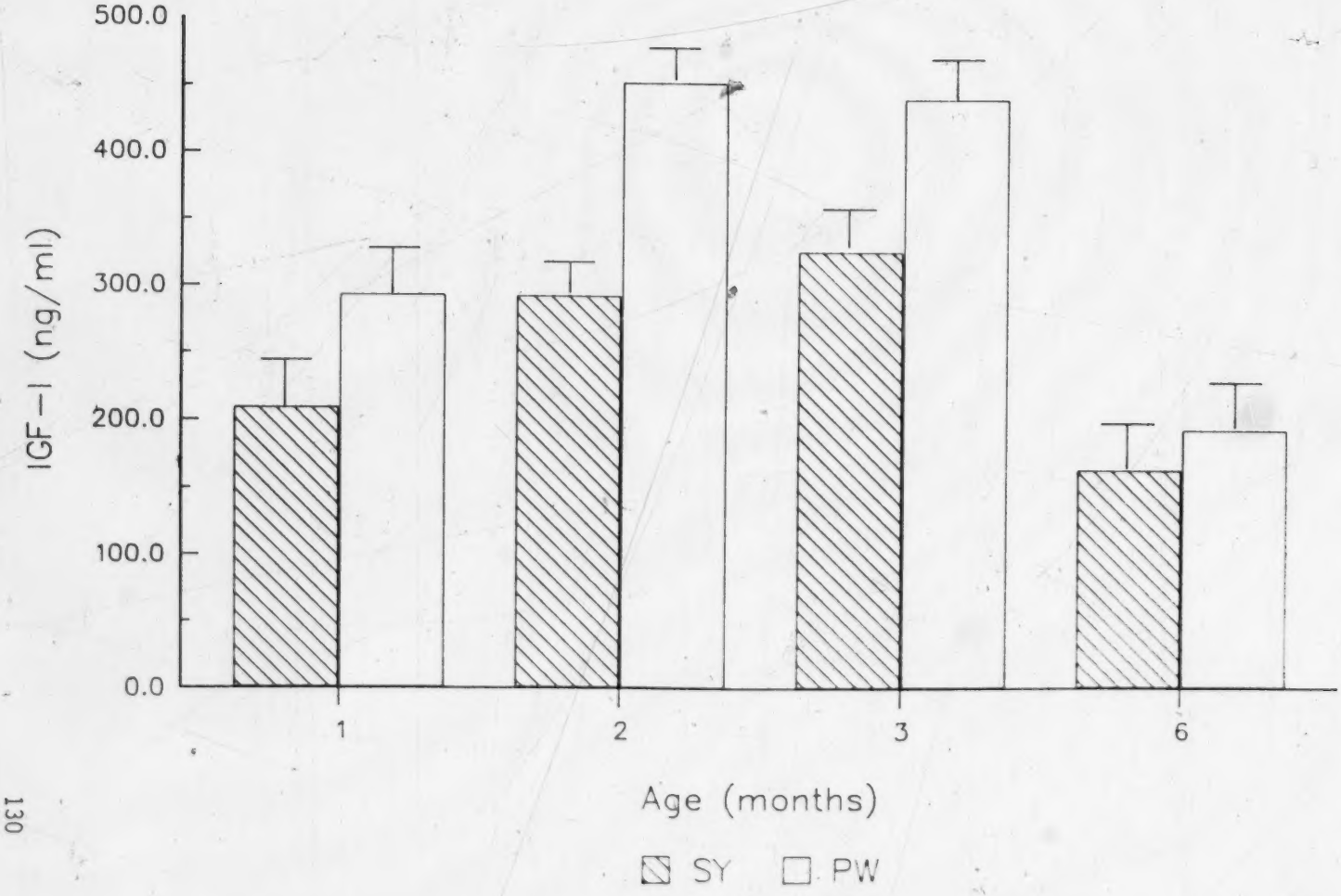

500.0
400.0
300.0
200.0
100.0
0.0
IGF-1 (ng/ml)
1
2
3
6
Age (months)
SY
PW

Figure V.2. Individual (PW, SY) and averaged (AVE; PW (---), SY (——)) plasma insulin-like growth factor I (IGF-I) concentrations (ng/ml) with body weight (kg) for animals of the pee-wee (PW) and beef synthetic (SY) strains at 1, 2 and 6 months of age (month 3 is indicated in AVE).

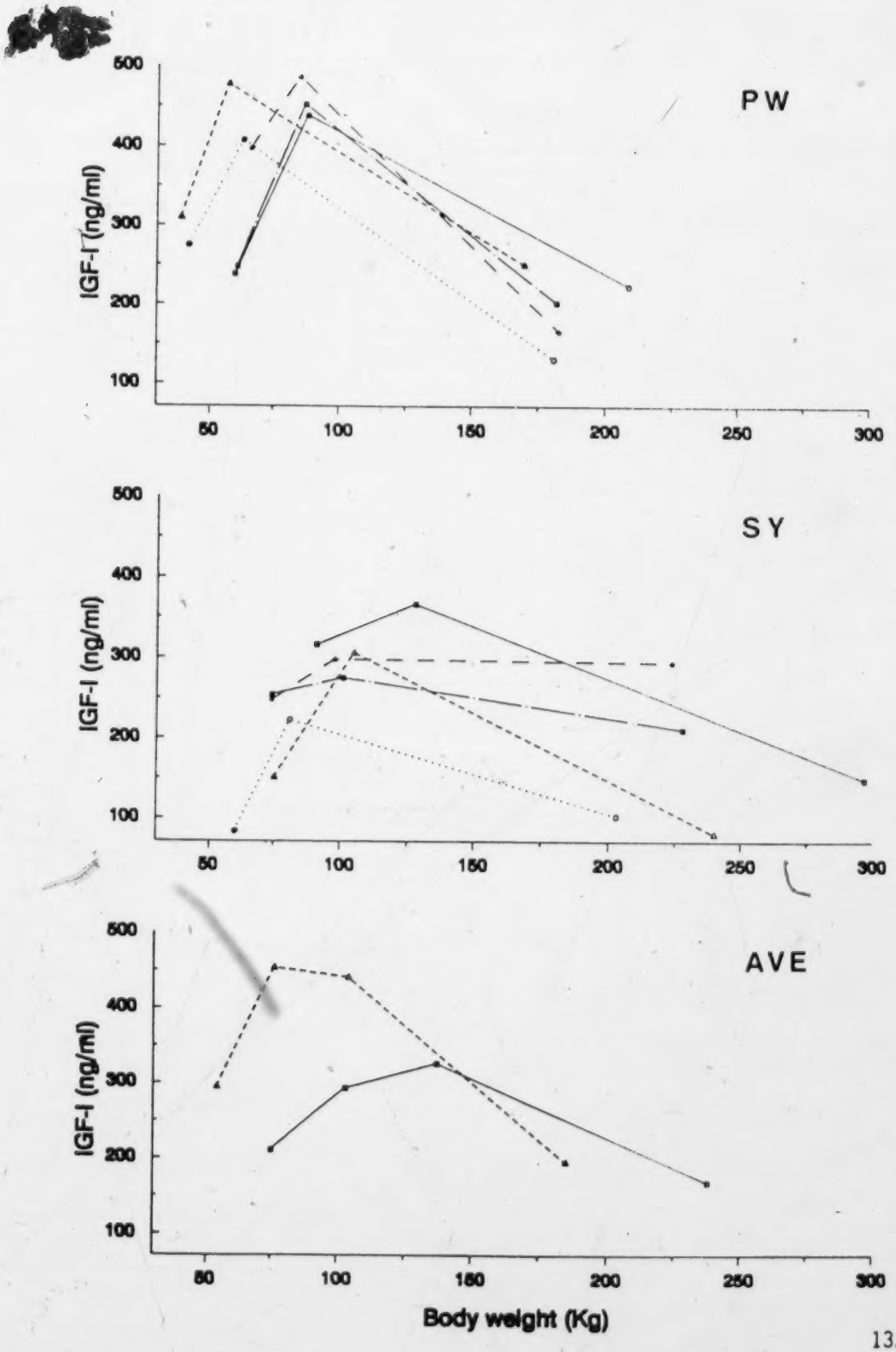
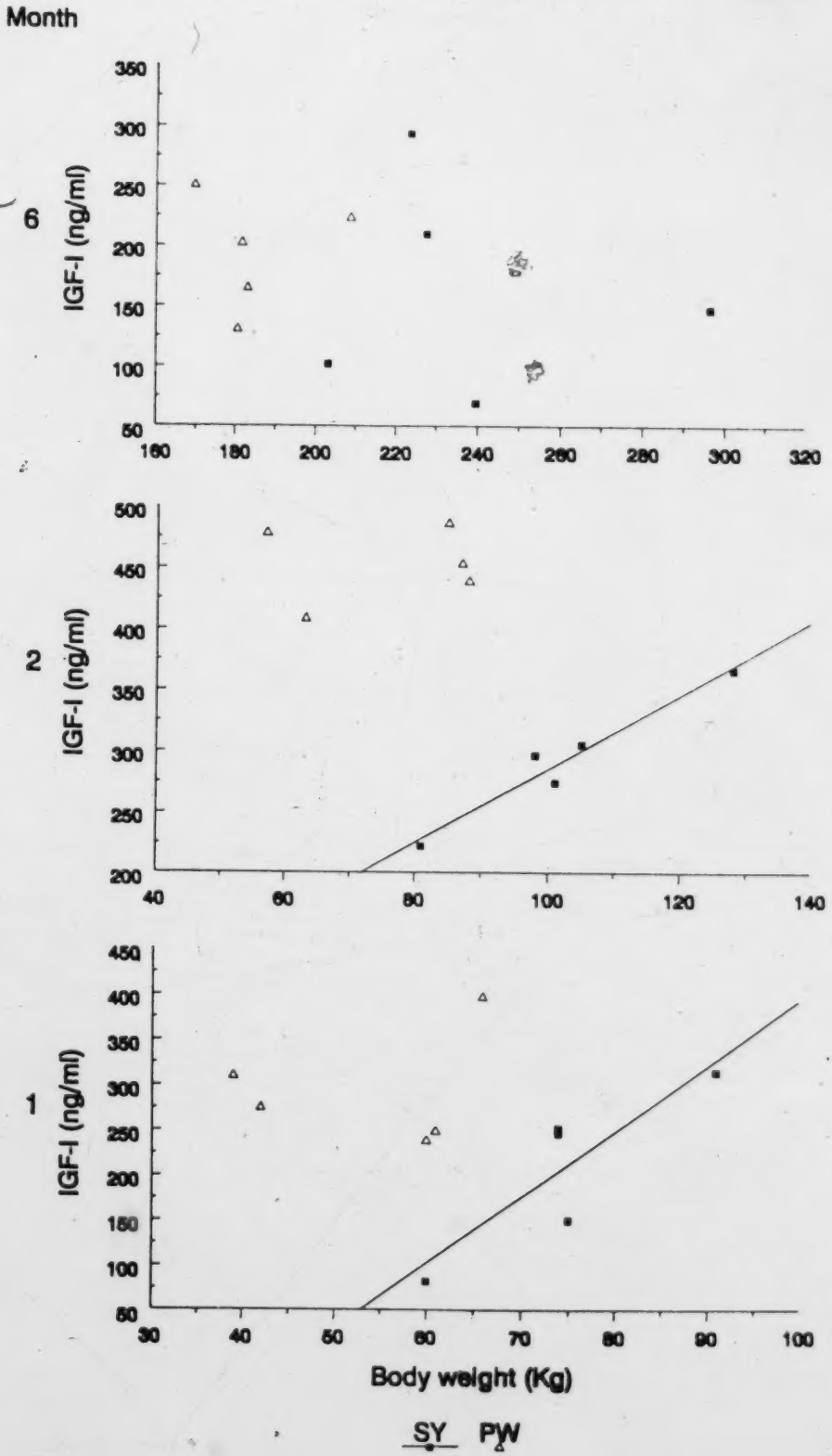

PW
SY
AVE
IGF-I (ng/ml)
500
400
300
200
100
50
100
150
200
250
300
Body weight (Kg)

Figure V.3. Plasma insulin-like growth factor I (IGF-I) concentration (ng/ml) with body weight (kg) for each member of the beef synthetic (SY) and pee-wee (PW) strains at 1, 2 and 6 months of age. Simple linear regression generates an adjusted r-squared of 0.6673 (p=0.05), 0.9304 (p=0.005) and -0.3197 (p=0.87) for SY, 0.2884 (p=0.76), -0.3108 (p=0.83) and -0.3302 (p=0.93) for PW at month 1, 2 and 6 respectively. Only regression lines of significance are drawn.


Month
6
IGF-I (ng/ml)
350
300
250
200
150
100
50
160
180
200
220
240
260
280
300
320
2
IGF-I (ng/ml)
500
450
400
350
300
250
200
40
60
80
100
120
140
1
IGF-I (ng/ml)
450
400
350
300
250
200
150
100
50
30
40
50
60
70
80
90
100
Body weight (Kg)
SY
PW

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VI. GENERAL DISCUSSION

Insulin-like growth factor I stimulates cellular proliferation and differentiation as well as exerts insulin-like effects on intermediary metabolism in a variety of cell types (Bradshaw and Sporn, 1983; Ewton and Florini, 1980; Hill, 1988). The possible involvement of IGF-I in several commercially important traits including liveweight gain (Blair et al., 1987, 1989) and muscle growth (Tollefsen et al., 1989), has led to interest in its potential to facilitate increases in the productivity of domestic cattle. The objectives of this thesis were to further clarify the role of IGF-I in growth and development, and to elucidate possible molecular heterogeneity contributing to the differential muscling phenotypes observed in PW, SY, and DM experimental animals.

Although the strong correlations between growth hormone levels, hepatic IGF-I content, and circulating IGF-I levels support the premise that liver is the primary target for the stimulation of IGF-I production (Goldstein et al., 1988; Schwander et al., 1983), multiple extrahepatic tissues have been shown to express this growth factor (D'Ercole et al., 1984; Han et al., 1987a,b; Murphy et al., 1987). The relative contributions of hepatic and extrahepatic IGF-I to its mitogenic activity are yet to be determined; however, it is tempting to postulate that multiplurent production of IGF-I may reflect a localized control of body growth in which autocrine and/or paracrine activities augment an endocrine homeorhesis. Hepatic tissue may have evolved to take advantage of its unique physiological and anatomical properties to sustain blood-born

concentrations of IGF-I. The liver is the body's largest compound gland and represents its principle metabolic organ. In humans, ~30% of the total cardiac output flows through the liver every minute (Guyton, 1981). Reminiscent of the hypophyseal portal circulation, the hepatic circulation is an exception to the usual arrangement of the systemic vasculature in that the liver is primarily perfused by blood from a vein (portal) rather than an artery. Since the portal vein conveys nutrients and hormones from the gastrointestinal tract, and since the nature of the hepatic sinusoid endothelial lining promotes rapid exchange of material between blood and hepatocytes, the liver is well positioned to coordinate a higher order limbic signal for IGF-I production (GH from the pituitary) with agents necessary to facilitate the growth response. Supporting this premise, both insulin (Canover and Lee, 1990) and nutrient balance (Davenport et al., 1990; Emler and Schalch, 1987) have been shown to regulate IGF-I gene expression in liver. The high volume of blood coursing through this tissue, together with the observation that hepatic output empties directly into the inferior vena cava, ensures a rapid dispersal of IGF-I and nutrients throughout the systemic circulation. In turn, IGF-I can fine-tune its blood-born production levels by exerting negative feedback regulation on GH expression (Yamashita and Melmed, 1986; Yamashita et al., 1986).

Trophic stimuli for specific cell types may enhance the activity of a sustained IGF-I concentration through the expression of autocrine and/or paracrine IGF-I. Northern blot hybridization analysis was used to demonstrate that IGF-I mRNA are expressed locally in bovine skeletal

muscle, liver, and pituitary tissues. Comparison of preliminary results with those values reported in the literature (see Figure I.1, I.2) suggested the potential for nonspecific hybridization of radiolabelled probe with cellular RNA. Such nonspecific hybridization may have promoted erroneous interpretation of IGF-I signals obtained previously, not only by Northern analysis, but also by dot blot, in-situ hybridization, and/or solution hybridization techniques. To alleviate this potential shortcoming, methodological validations of specific IGF-I transcripts were undertaken (Chapter II). Hybridization of poly A^+ RNA with a combination of distinct probe types under conditions close to the predicted Tm of the probe/message hybrid, provided a reproducible and sensitive means to interpret specific IGF-I transcripts. Through this approach, it became apparent that IGF-I mRNA displayed tissue and phylogenic heterogeneity (Chapter III). Transcripts of 7.7kb and 1.1-0.8kb were detected in both bovine and rat liver, while bovine liver displayed additional IGF-I signals at 10.3kb and 3.5kb. Bovine skeletal muscle and pituitary tissue exhibited IGF-I messages at 9.4kb and 7.7kb. Analogous to its primary ligand, type I IGF receptor mRNA also demonstrated expression heterogeneity across and within species. Notwithstanding the absence of specific changes in size distribution of the receptor transcripts, differences were apparent in hybridization signal intensities between rat liver, bovine liver, and bovine muscle. The 11.0kb and 6.1kb signals were the strongest and most well defined in bovine muscle, followed by bovine liver and then by rat liver. Rat liver transcripts of estimated size 4.4kb and 3.1kb appeared more intense than those detected in bovine muscle or liver respectively. The variation in abundance and/or species of IGF-

I and type I IGF receptor mRNA amongst different tissues suggests tissue and phylogenic specific differences in IGF-I gene expression, mRNA precursor processing, and/or mRNA stability. The different lengths of mRNA observed could also represent discrete translation termination products resulting from specific signals encoded in the mRNA.

To examine whether heterogeneity in transcript expression and/or processing of IGF-I and/or type I IGF receptor mRNAs could contribute to the differential muscling exhibited between PW, SY, and DM cattle, investigations analyzed skeletal muscle poly A^+ RNA by Northern hybridization with riboprobes corresponding to each polypeptide (Chapter IV). The data obtained demonstrated that species of IGF-I and type I IGF receptor transcripts did not differ amongst the 3 strains, nor did they differ over the first 6 months of postnatal development. However, the intensity of all transcripts progressively increased over the investigative period. These findings suggest that the mRNA for IGF-I and type I IGF receptor may be developmentally regulated at the level of gene expression and/or mRNA stability. Although the absence of heterogeneity in mRNA signal intensities and size distribution imply that these mechanism do not directly contribute to the differential muscling phenotypes of the 3 strains, it is conceivable that the ontogenic patterns of expression represent a common mechanism to effect cell responsiveness. Correlating expression level changes with other endocrine and metabolic fluxes during the prepubertal period could provide insight into this regulation. Further studies are required to clarify processing modification, stability, translatability, and physiological role of the

alternative IGF-I and type I IGF receptor transcripts. Moreover, whether the peptide products of these alternative mRNAs interact differently with each other, or with IGF binding proteins, or whether the alternative E peptides of IGF-IA and IGF-IB have a role in differential muscling, remains to be explored as well. Exon specific IGF-I probing of cellular RNA could help to resolve this last point.

There is a growing body of evidence linking circulating IGF-I levels to growth rate and body size in domestic cattle (Anderson et al., 1988; Lund-larson et al., 1977; Ronge and Blum, 1989). As such, and since PW bull calves were significantly lighter and had a significantly lower ADG than SY, it was assumed that PW animals would exhibit lower serum IGF-I concentrations than SY. Nevertheless, hormonal data demonstrated that PW calves had significantly higher mean circulating IGF-I levels than did SY animals at all sampling periods (Chapter V). It was further evident that during the same period, not only did IGF-I levels not show a positive linear correlation with weight across PW and SY strains, but that such correlations were absent within strains as well. The relationships between serum IGF-I and body weight were best described by downward facing parabo'ic curves. These ontogenic expression profiles, although in keeping with the general serum IGF-I patterns observed in other mammals (Bala et al., 1981; Gluckman and Butler, 1983), displayed a species specific chronological component. This component aligned well with the design of retrograde change apparent in the diphasic growth pattern of several bovine muscle groups (Butterfield and Berg, 1966a,b). It is possible that the *high impetus* phase of muscle development parallels an

increase in circulating IGF-I levels and the *low impetus* phase commences as serum levels decline.

Although not reflected in plasma values of the growth factor, circulating IGF-I, in combination with modulators of cell responsiveness, may play an important function in growth regulation. There exists a number of potential mechanisms by which cellular sensitivity to IGF-I can be governed; the most relevant of which to this discussion is the activity of the IGF binding proteins. In all interstitial and extracelluar fluids analyzed, ~99% of the IGF-I circulates noncovalently complexed to specific high affinity proteins (Clemmons et al., 1989; Zapf and Froesch, 1987). Two major forms of IGF BP with apparent molecular weight of 150kda and 25-45kda have been identified in human plasma (Furlanetto, 1980; Hintz and Liu, 1977). The majority of IGF-I is bound to the 150kda BP, which similar to IGF-I, is regulated by GH. The low molecular weight BPs are considered GH-independent (Zapf et al., 1980). The physiological functions of circulating binding proteins remain unclear. Present evidence suggests that IGF-I/BP conjugates do not bind to IGF-I receptors. Nevertheless, inhibition (Rutanen et al., 1988) as well as enhancement (Elgin et al., 1987) of IGF-I activity have been attributed to these proteins. They modulate the actions of IGF-I in vivo by inactivating acute insulinomimetic effects characteristic of free IGFs (Binoux et al., 1986), while at the same time increasing the growth factor's half life (Hintz, 1984), and, by virtue of carboxy terminus Arg-Gly-Asp tripeptide sequences (Ruoslahti and Pierschbacher, 1986), facilite a target tissue micro-reservoir of IGF-I. Several cell types have been shown to secrete

BPs (McCusker and Clemmons, 1988; Scott et al., 1985). Accordingly, it is feasible that acute regulation of IGF-I activity may partially occur not only through mediation of ligand expression levels, but additionally through binding protein governance. Detailed ontogenic studies on the physiological role of BPs in modulating IGF-I action in PW and SY animals, are required to determine if these proteins contribute to the relationships between IGF-I levels and body weight that are observed in these strains.

It is evident from this work that although IGF-I and type I IGF receptor expression are dynamic characteristics of postnatal growth, heterogeneity in these determinants does not appear to be involved in the differential muscling of PW, SY or DM strains of domestic cattle. In the postnatal animal, there may be the potential for an interplay of autocrine/paracrine and endocrine actions of IGF-I. Not mutually exclusive, which mode of action emphasized would vary depending on developmental and physiological state. The preliminary data accumulated suggest that neither circulating levels of IGF-I nor skeletal muscle levels of IGF-I or type I IGF receptor mRNA may be indicators of growth potential in cattle during the first 6 months of development. The utility of these genetic parameters for selection of breeding animals or transformation of embryos will only be established through further investigation.

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